# EMBO PRACTICAL COURSE
# ON
# Molecular and Genetic Tools for the Analysis of Medaka and Zebrafish Development

## European Molecular Biology Laboratory, EMBL HEIDELBERG
## July 21st – 31st, 2002

Design by Petra Riedinger, EMBL, Heidelberg


Organized by:
Jochen Wittbrodt (Heidelberg)
Felix Loosli (Heidelberg)
Manfred Schartl (Würzburg)
Wolfgang Driever (Freiburg)
Akihiro Shima (Tokyo)
Monte Westerfield (Eugene)

Helped by the Wittbrodt lab
Alexander Arenz, Matthias Carl, Filippo DelBene, Clemens Grabher, Erika Grzebisz, Annette Krone, Felix Loosli, Lucia Poggi, Rebecca Quiring, Martina Rembold, Beate Wittbrodt

and
Emma Fassmann and Laura Vinti


# COURSE MANUAL

This manual contains protocols and experimental procedures for the experiments to be performed at the EMBO course in Heidelberg. Protocols have been contributed by the instructors. Additional, relevant protocols have been included in this manual from previous EMBO courses and from the Zebrafish Web Site at the University of Oregon (http://zfish.uoregon.edu/zf_info/zfbook/zfbk.html).

We also included some protocols which will not be used during the course, but may be relevant to you once you are back home and want to perform real experiments. Many of these protocols are unpublished, lab based methods.

Contributions by:

L. Bally-Cuif
M. Carl, Heidelberg
W. Driever, Heidelberg
M. Furutani-Seiki, Kyoto
C. Grabher, Heidelberg
T. Henrich, Kyoto
R. Ho, Princeton
Y. Hong, Singapore
D. Kane, Rochester
A. Krone, Heidelberg
F. Loosli, Heidelberg
T. Matsuoka, Tokyo
H. Mitani, Tokyo
I. Nanda, Wuerzburg
K. Naruse, Tokyo
C. Neumann, Heidelberg
R. Quiring, Heidelberg
E. Raz, Goettingen
M. Rembold, Heidelberg
M. Schartl, Würzburg
A. Schier, New York
A. Shima, Tokyo
A. Shimada, Tokyo
L. Solnica-Krezel, Vanderbilt
B. Thisse, Strasbourg
Ch. Thisse, Strasbourg
H. Wada, Tokyo
Y. Wakamatsu, Nagoya
C. Walker, Eugene
M. Westerfield, Eugene
C. Winkler, Wuerzburg
B. Wittbrodt, Heidelberg
J. Wittbrodt, Heidelberg

Supported by: EMBO - European Molecular Biology Organization

The organizers gratefully acknowledge support from the following companies:

Eppendorf

Zeiss

Intavis

Müller und Pfleger

Leica

Biozym

New England Biolabs

Clontech

Millipore

Roche

Promega

Qiagen

Stratagene

Invitrogen

Harvard Apparatus

Greiner

Inter Ryba (Hamburg)

# 1. Obtaining Fish Embryos

## A Fish Colony

A variety of set-up options currently exist for fish facilities. On a small scale, it is possible to maintain fish for a constant supply of good eggs using standard aquarium tank set-ups obtained through a pet shop.

For zebrafish the biggest problem may be to get young and good fish for egg production. In the US, Ekk Will Waterlife Resources (Symmes Road, Gibsonton, FL 33534-9714 phone: 1-800-237-4222) have provided us reliably with good breeders. In Europe, investigate with larger retailers.

For Medaka the best source of well producing heathy adults is Carolina Biological supply (https://www3.carolina.com/onlinecatalog/default.asp).

Depending on your financial frame and caretaker support, different systems have worked out successfully for maintaining medaka and/or zebrafish colonies.

For a medium sized facility of 100-400 fish tanks, we find the Z-module systems from Marine Biotech (Marine Biotech, Inc.; 54 West Dane Street; Unit A; Beverly; Massachusetts 01915; USA; Telephone: +01 978 927-8720; Telefax: +01 978 921-0231) very easy to set up and run. Modules of up to 84 tanks can be combined to larger units. Setup is provided by the company, or can also be easily performed by a local workshop.

For larger systems, three companies have provided excellent solutions: Müller & Pfleger GmbH (Wiesenstrasse 27-29; Postfach 1234; D-67806 Rockenhausen; Germany; Telephone: 011-49-6361-92160; Telefax: 011-49-6361-921628, Aquarienbau Schwarz (Fabrikweg 8; D-37075 Göttingen; Germany; Telephone: 0551-33340; Telefax: 0551-376533), as well as Marine Biotech.

The system we are using is based on recirculating water flow. Water from a lower biofilter reservoir is pumped to an elevated reservoir above the level of the aquaria. The pump is a bit stronger than needed, and some water is always draining back to the lower reservoir, such that water levels in the top reservoir is constant. On its way to the top reservoir, water can be filtered through fine particle filters (Rainbow, USA), and sterilized using UV irradiation bulbs. Water from the top reservoir is fed to the tanks, such that the water is exchanged 2-3 times per hour (zebrafish) or once in two hours (medaka) in each tank. Tank sizes range from 2-3 liter (mouse cages size A; Tecniplast Deutschland GmbH; Hettenstr, 18, D-82383, Hohenpeissenberg; for up to 10 fish), 5 liter (glass tanks, up to 20 fish) to 12 to 15 liter tanks (glass tanks, up to 50 fish). Water from the tanks flows over into a draining system of pipes that delivers it to the top of a biofilter dry-wet trickle unit. Filter pads (polyester foam egg crate padding as used in bedding materials and other types of foam pads) in 6-8 drawers remove the solid waste (top drawers) acting like particle filters, and are substrate for biological filtration (denitrification). For zebrafish day about 10% of the total volume of the facility water is replaced by adding reversed osmosis purified water, for medaka 10% exchange per week is optimal (and zebrafish used in the course are kept under those conditions). To reconstitute salts to a level acceptable fish, we mix charcoal filtered tap water and fully deionized water (see below).

System parameters are: temperature 26.0-28.0°C (controlled by room air temperature); salinity 0.03 - 0.04% (conductivity: 0.2 mS); pH between 6.8 and 7.2; ammonia and nitrite below 2 ppm; and nitrate below 60 ppm. Fish are kept at a 14 hours light / 10 hours dark cycle (8.00 a.m. to 10:00 p.m.). Tanks are kept clean and debris are syphoned off.

The cleaning regime for our fish systems is as follows; the process works in a 6 week cycle. From the time that the system has been set-up, 3 weeks from this time, a 20% water change is to be performed. This is in addition to the water that will be replaced due to evaporation on a daily basis. Then 3 weeks after the water change, ie: in the 5th week after the system set-up, a filter clean should be carried out. By filter clean, this basically means a thorough cleaning of every part of the system. This should include removing all the filters from the systems and eliminating all dirty particles from all filters. A lot of debris will form in the tanks as well as in the filter housing itself, this should be removed/ syphoned off at the same time. Then, once again, 3 weeks after the filter cleaning, a 20% water change should be performed etc etc. It is imperative that this cleaning schedule is maintained, the well being of all fish depends on a well kept aquarium!

## Salt composition of water in fish facility

Our fish water is based on a 80% distilled water and 20% tap water (charcoal filtered) mix resulting in a conductivity of 150 µS/cm. Our system automatically mixes 4000 liters to this conductivity. The water is complemented by the additon of 5g/100L Sera Mineral Salt (Sera GmbH; P.O. Box 1466; D-52518; Heinsberg; Germany; Telephone: +49 2452 912615; Telefax: +49 2452 5922), trace elements using 100mg/L Mikrosal supplement (H. Brustmann GmbH & Co. KG; P.O. Box 1129; D-65370; Oestrich-Winkel; Germany; Telephone: +49 6723 91767; Telefax: +49 6723 7772) and Ferrogan fluid; 2 drops/50L (H. Brustmann; see above). Due to the hardness of our tap water, it would be advisable to check with your local water board to assess the hardness of your local tap water, once this is known, you can play with the two until the desired mix (conductivity) is obtained. The pH in the recirculating water system equilibrates to about 6.9 due to acid production in tanks and filters. We experienced that the addition of filtered tap water dramatically improved the buffer capacity as compared to fully artificial water (even if we added buffering components there). Water contitioner such as tetra aqua safe did not have additional beneficial effects and we avoid it completely.

## Feeding Fish

We feed hatched artemia nauplii (Western Brine Shrimp International, Inc.- Utah, “Red Dragon” & Inter Ryba GmbH- Germany, “Silver Star Artemia”- Product of USA ) and flake food (JBL or Tetra). For raising larvae, Tetra produces a food, AZ 100, which is not commercially available so far, but can be obtained from the company:

Dr. H. Kürzinger; TETRA WERKE; Herrenteich 78; D-49304 Melle, Germany;

fax: +49 5422-42986. AZ 100 can be fed from day 5 of development onwards.

Recently we had excellent success with a liquid baby food (Baby Star, Inter Ryba, Dr. B. Jung, Tannenkamp 12, 27404 Zeven, Germany, phone: +49 4281-958833, fax: +49 4281-958836) that comes in different sizes and can be better adjusted to the size of the growing young fish. The food does not float on the surface of the baby tanks, thus no decay, less cleaning! As the food is suspended in the water, much the same as hatched artemia, the food is eaten quickly by the larvae.

Adult fish are fed three to four times with artemia nauplii and Tetra-min dry food.

## Setting up crosses to obtain eggs by natural breeding

(W. Driever)

Eggs from zebrafish can be obtained by either placing a breeding trap into a tank with many fishes, or by setting up a defined cross between a male and a female in a single trap on shelves in the fish room.

Genetic crosses between single females and males are productive if the fish are older than three months. You have a good chance to get large egg clutches when you follow most of these rules:

- use males and females once every one to two weeks
- use males and females of approximately the same size (of course, females are always slightly larger)
- handle fish for crosses very carefully
- set up crosses in the late afternoon or evening before the light turns off (14 hrs. light / 10 hrs. dark cycle) Make sure that you do not transfer debris or food from the tank into the breeding trap, which makes the water turn bad overnight and the fish suffer or die!!!!

### Set up the cross as follows:

1. Assemble a breeding trap: put a white plastic container (with the steel mesh) into a clear one. Fill with fish water so that the water level is about 3 cm above the steel mesh.
2. Transfer fish from tank to one mouse cage. Separate males and females from the mouse cage into two separate mouse cages. Check the sexes by looking from the side at the mouse cage, while it is placed on a dark surface (when melanophores are dilated, males and females are easier to distinguish)
3. Add the male and then the female Take nets made of white cloth net, which is softer than the green nets and better for small fish. (In a lab setting, you would use a fresh net for each new tank you take fish from, so not to transmit any putative diseases. Used nets get autoclaved). Cover the breeding trap with a lid, and place the whole trap on a black surface (bench or shelves).

   Now your fish will or will not lay eggs (for wild type, between 5 and 8 out of 10 crosses might lay eggs). On the next day: If you need to carry the trap, don't make any rapid movements: the eggs will whirl up and the fish will eat them! For all crosses that gave eggs, prepare a second label and stick it to the breeding trap: one label will go with the fish, the other with the eggs! This prevents mix ups if you have to keep the parents for identification of genotype / new mutations.

5. Net the fish into new mouse cages before you put them into their appropriate containers or tanks. This allows to carefully check the sexes and to make sure that you do not mix up fish. Check the sexes by looking from the side at the mouse cage, which is placed on a black surface.
6. Remove the top of the trap.
7. Recover the eggs by filtering the water from the breeding trap through a plastic tea sieve (I do this with the sieve placed in a large petri dish, such that the stream of water can not damage the eggs).
9. Transfer eggs from sieve into 10 or 15 cm plastic Petri dish (with help of spray bottle). Fill with egg water.
10. Take a Pasteur pipette with a wide opening (VWR brand, or cut and flame polished, which is better) and pipette the good, fertilized eggs carefully into a fresh dish with egg water. Make sure that the opening of the pipette is large enough: the eggs should fit through easily, so that

the chorion is not hurt. When you pipette eggs: never fill more than 50 eggs into the pipette; when you want to release the eggs again, first suck in some more water (so that the eggs get whirled up) and then release them: otherwise they clog the pipette and they will be distorted if you try to press them out. Label the dish with the second tape you prepared!

11. Transfer to new Petri dish with methylene blue saline
12. Never put more than 200 eggs into a 15 cm dish with 200 ml of egg water (50 eggs in 10 cm dish with 30 ml egg water): they will not have enough oxygen, and bacteria growing on the chorions might later suffocate the fish.
13. Label the bottom part of the dish with a tape (at that stage you can write anything on it, but the date and the genotype of the parents are certainly helpful).
14. Remove dead / black eggs in the evening and on the following morning: fungus will grow on the dead eggs and suffocate the good ones.

---

# Obtaining medaka embryos

(E. Gzebisz and J. Wittbrodt)

## Mass production of embryos

The natural habitats of the medaka are the rice fields of South east Asia. In the laboratory these fish are extremely hardy and have no high demands for water quality, food or specific temperatures. They tolerate direct sun light, massive growth of algae and changing temperature levels. Their natural spawning season is between June and September with spawning occurring at dawn. One female usually produces more than 3000 eggs per breeding season. Under laboratory conditions daily egg production can be induced throughout the year by an artificial photoperiod of 14 hrs light/10 hrs darkness. Three factors are instrumental for successful embryo production in the laboratory. Most important is the high intensity of light applied for a specific time. Furthermore, optimal water temperature (28°C) and sufficient food supply (3 times daily; tetramin and/or brine shrimps) guarantee good egg production. A good and healthy adult female produces between 20 and 50 eggs per day in a laboratory tank for up to 3 months. After three months males and females should be returned to a resting cycle (12hrs light/12hrs darkness; low food supply) to prevent exhaustion.

Males and females can be easily distinguished by their differently shaped anal and dorsal fins. The male dorsal fin has a clearly visible deep notch between the last two rays. The male fins, both dorsal and anal, are larger compared to female fins. Those have a round shape with smooth margin and do not have notches.

To obtain egg production in the laboratory, young medaka fish are kept after hatching under resting conditions at high density in large containers for up to 8 weeks after hatching (100 fish in a 50 liter tank). Young fish just after hatching are fed with rotifers, infusoria and/or powdered tetramin. Two weeks after hatching fish are fed with brine shrimps and tetramin. No filtering or air bubbling is required and app. 1/10 of the water is exchanged once a week. After 8 weeks (the generation time of medaka is 8-10 weeks) the light phase is changed to 14hrs and the fish are fed at least 3x daily (brine shrimps and/or tetramin). Under these conditions after 1-2 weeks the females start spawning with about 5-10 eggs per day. Males and females can now be separated for several days before bringing them together for the production of large numbers of eggs.

For mass production adult fish are kept at high density in large plastic containers with plants but without filtering and air bubbling. Medaka females carry clusters of eggs at their belly for several hours before they are stripped off at plants in the tank. After onset of egg production (2 weeks after changing the photoperiod) all plants with adherent eggs are removed from the tanks with the adult fish and put into fresh tanks with aged, algae green tap water and free of snails or adult fish. After 2 weeks medaka fish will hatch and can be fed with rotifers.

## Production of embryos for microinjection

About 6 adult fish (5 females, 1 male) that have recently started to produce eggs under the reproductive cycle are transferred to a 7 l tank. Daily spawning takes place within the first 30 minutes after the light is set on. The eggs stick together through hairy filaments and attach to the mother's belly for several hours. Fertilized eggs can be obtained by carefully stripping off the egg clusters from females using a bend preparation needle. Single eggs are obtained either by rolling egg clusters on a whatman filter paper or by using forceps to remove hairy filaments under a stereoscope.

Injected embryos are transferred to Embryo Rearing Medium where they will hatch after 7-9 days. Dead embryos are removed daily. It is not necessary to change the medium before hatching except if there are signs of bacterial growth.

Embryos isolated from females during the first 60 minutes after the light is switched on, are still in the one-cell stage and need app. another 30 minutes to complete the first cell division. This gives enough time for microinjection into the 1 or 2-cell stage. However, it is possible to slow down development of early medaka embryos by incubating the eggs at 4°C for several hours without any problems with respect to lethality or abnormal development. Embryos that are past midblastula stage can be stored for several weeks at 4°C to arrest development without any damage. The cold tolerance together with a developmental time of minimum 7 days also allows mailing of large numbers of medaka embryos by mail in Embryo Rearing Medium (ERM) in an Eppendorf tube.

Eggs from medaka can be taken every day from a coupling. It is also possible, and widely used, to set-up one male with up to 5 females. As the eggs remain attached to the belly of the female after fertilization, there is no need to house the matings in special cages.

- the medaka adults will become sexually mature at around 3-4 months of age, if you push them this time can be reduced to as little as 2 months.
- When collecting the embryos handle the fish very carefully as not to cause any injury or too much distress!

1. Once you have decided the strain/s of fish that you want to use, you can go ahead and select a producing female/s and a fertile male for the mating set-up.
2. Once this has been done, the chosen fish can be placed together in a tank. Both glass and plastic tanks can be used for matings.
3. The fish usually prefer a couple of days to adjust to one another, but more often than not the females will begin to produce straight away. Spawning usually takes place at dawn, but this varies from strain to strain. Before spawning, the male displays a characteristic sexual manoeuvring (dancing). The male approaches the female and performs swift circular movements around the female. Once mating is complete a cluster of eggs remains attached to the belly of the female for some hours, they hang from the oviduct by fine threads attached to the chorion of the eggs. Those eggs that appear milky in colour may not be fertilised.
4. Once you see that the fish have mated, carefully remove the female from the tank with a fine net. Using either your fingers or, preferably, a egg hook (metal rod with slightly curved wire at

one end, please ensure that the hook is completely blunted at curved end to avoid injury to the female) gently scrape the eggs away from the females abdomen onto the hook.

5. Tranfer the cluster of eggs into a petri-dish with 1x hatching solutiuon or 1x ERM, dependant on the use of the embryos afterward. If screening the embryos for flourescence, then it is better to use the ERM solution, the methylene blue in the hatching solution represses the expression of flourescence somewhat.
6. Label the top of the pertri-dish with the name of the strain and the date of collection, together with any additional information that may be useful.
7. It is advisable to seperate the eggs from one another. This can be done in two ways, firstly, simply using your index finger, gently place the finger in the top of the cluster and move your finger in a circular motion. (Be careful not to apply too much pressure to the eggs as this will cause them to burst!) You will see the eggs start to seperate from one another. The alternative is to place the egg cluster on a piece of filter paper or tissue and seperate the eggs using your index finger or a pair of fine forceps. The forceps need to be distiguisable between those used to handle living embryos and those for dead embryos. (The easiest way to do this is to label the “dead” forceps with some red tape and the “living” with some green tape.)
8. Once the eggs have been seperated remove all dead and injured embryos from the petri-dish using forceps. (Dead/injured embryos decay and will cause bacteria to grow in the dish.)
9. Remove all debris (ie. faeces, snails, algae) from the perti-dish using either the forceps or a disposable pastuer pipette.
10. Drain off the original hatching solution or ERM. This can be done successfully by holding the lid of the petri-dish tightly and pouring off the liquid (the eggs will remain in the dish). Once the liquid has been removed, refill the petri-dish with hatching solution or ERM (the best is to use a spray bottle, as this helps you to remove the eggs that may have attached to the side of the dish without damaging them). Alternatively, you can take up the eggs with a pastuer pipette and transfer them to a newly filled petri-dish. Make sure not to use the pipette for different stocks and strains!
11. Never put more than 200 eggs into a 15 cm dish with 200 ml of hatch solution (50 eggs in 10 cm dish with 30 ml hatch sol.): the eggs will not have enough oxygen, and bacteria growing on the chorions might later suffocate the fish.
12. Check the petri-dish on a regular basis to ensure that no bacteria is growing and to remove any embryos that may have died, this helps the healthy embryos to develop in the best conditions possible.

## References

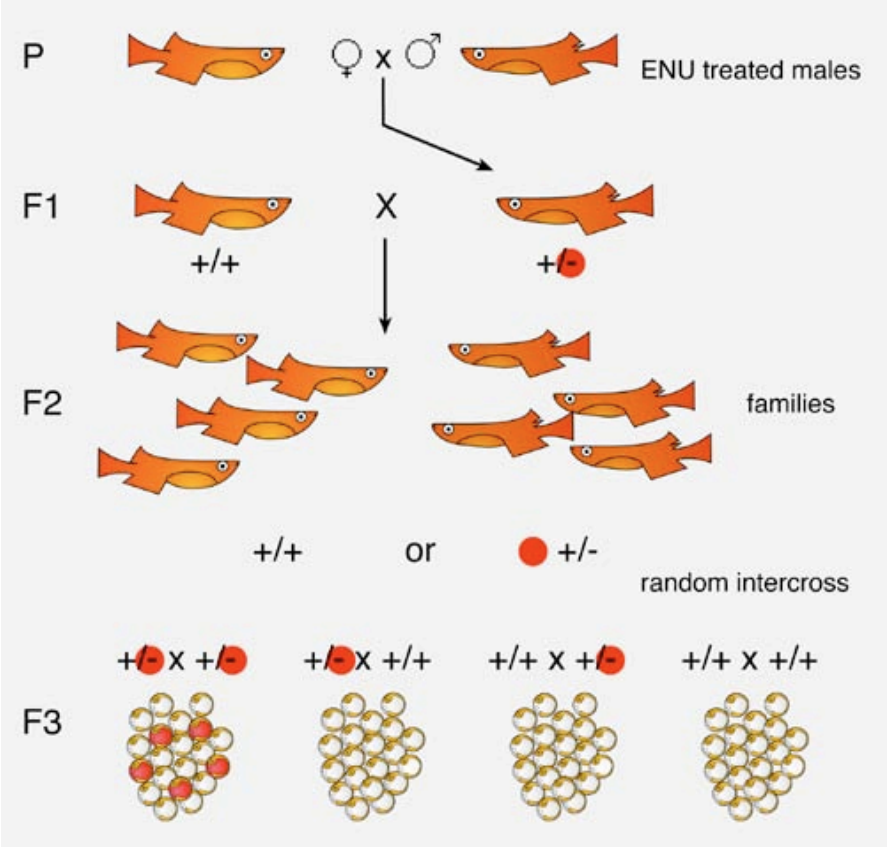
Kirchen, R.V. & West, W.R. (1976). The Japanese medaka - its care and development. Carolina biological supply Company. Burlington, North Carolina.

Yamamoto, T. (1975). Medaka (Killifish) - Biology and Strains. Keigaku Publishing Company, Tokyo, Japan.

# 2. GENETIC APPROACHES

## 2.1 Mutagenesis screen in medaka

(contributed by M. Furatani-Seiki)




The experiment of a mutagenesis screen in Medaka, consists of 3 major steps such as (1) mutagenesis of G0 male fish, (2) preparation of F2 families by two generation in-crossing and (3) screening of embryos from pairs of F2 families (Fig.1). Since the fist and the second steps take at least 9 month before you can start actual screening, it is recommended to make a plan about the time table, the scale of the screen (limited by the space of the fish facility or the speed of the screen) before you start a screen

Although F1 fish is crossed with wild-type fish to produce F2 families in this scheme, we usually make crosses within a F1 family.

Fig1. The three generation crossing scheme to detect recessive mutations (taken from Loosli et al., 2000)

### Experimental rationale

The chemical mutagen, N-ethyl-N-nitrosourea (ENU) can induce mutations in spematogonia and differentiating male sperm cells at the time of ENU treatment of the male fish. Based on the results of the study on cell population kinetics of spermatogenesis of the Medaka (Egami and Hyodo-Taguchi, 1967), maturation stages at the time of mutagenesis are judged according to the day of fertilization after mutagenesis as follows:

1 - 3 days after treatment; sperm

4 - 9 days after treatment; spermatids

10-15 days after treatment; spermatocytes

16-29 days after treatment; differentiating spermatogonia

30 or more days after treatment; stem spermatogonia

It is reported that ENU induces point mutations in spermatogonia but deletions or translocations in differentiating germ cells in zebrafish. Moreover, F1 fish become mosaic for induced mutations when fertilized with those sperms where mutations were induced at differentiating stages. As this makes the screen for the phenotype of mutations after 2 generations less efficient, mating of ENU treated G0 males with wild-type females to make F1 progenies should be started after 1 month of ENU treatment.

ENU mutagenesis is performed by the incubation of males in a ENU solution. The efficiency of mutagenesis is determined using specific locus test. For proliferating germ cells, ENU is an effective mutagen, as indicated by the frequencies of non-mosaic mutant progeny at four different pigmentation loci (Solnica-Krezel et al, 1994, Loosli et al., 2000). Several mutagenic regimens that varied in either the number of treatments, or the concentration of ENU were studied to achieve an optimal ratio between the mutagenicity and toxicity. In zebrafish, the two most mutagenic regimens: 4 x 1h in 3 mM ENU or 6 x 1h in 3 mM ENU, the minimum estimate of frequencies of independent mutations per locus per gamete was $0.9 - 1.3 \times 10^{-3}$. There was a more than 10 fold difference in the relative mutability of the least mutable locus brass and the most mutable tester locus sparse. ENU-induced mutations at the albino locus exhibited a range of hypomorphic phenotypes, indicating that the induced lesions were likely to be intragenic. They demonstrated that the mutations induced by ENU are transmitted to offspring and that they can be recovered in a two generation screen. The effectiveness of mutagenesis measured by the specific locus rate of $1.2 \times 10^{-3}$ approximately corresponds to two embryonic lethal mutations per single mutagenized genome. The high rates of germ-line mutagenesis achievable with ENU will allow saturation mutagenesis screens to identify genes involved in a variety of aspects of Medaka development.

During the EMBO course, the experimental setup will be demonstrated, but we will not actually use ENU: the course is too short to properly evaluate the experiment. In case you will actually engage in an ENU mutagenesis experiment, this will likely be one of the most dangerous (scientific) experiments in your life.

## Please follow these SAFETY INSTRUCTIONS

ENU can penetrate the skin and is an extremely active mutagen and carcinogen. Extreme care is necessary when handling this substance!

ENU is relatively stable in aqueous solutions, but gets inactivated in: 10% Sodium thiosulfate solution, pH adjusted to about 10 with sodium hydroxide.

Just to give you an idea, here are some half-life data:

ENU in Thiosulfate, pH >9 at 20 Celsius 0.05 hours

ENU in water: at 20 Celsius 1.6 hours

ENU in water is less stable, but at acidic pH it can be stable for a long time!

For ENU, buy the 1 g sealed bottle only (SIGMA, Isopac bottles). For consistency in your experiments, buy one batch such that you have a sufficient amount of ENU for one set of experiments. Never try to handle ENU powder (or you risk your life and your will-be kid's genes!). Handle the chemicals in a chemical hood only! And: perform all the mutagenesis in a chemical hood and inactivate the mutagen there, too. Under no conditions should any trace amount of mutagen have a chance to leave the chemical hood. Always label the area where you do mutagenesis very well, so that nobody will enter by accident!

Cloth: always wear lab coat and gloves when handling these chemicals. Of course, wear shoes and long pants to reduce risk of exposure.

Wear two pairs of gloves when handling mutagen solutions: in case of a spill, you can take off one pair without risking to expose bare skin! Cover the workplace with plastic backed paper. Dispose every single piece that had contact with the mutagen (pipette tips, fish nets, gloves etc.) into the thiosulfate solution.

Inactivate the mutagen solutions (where the fish swam in) in thiosulfate, too, and submerse the flasks in the bath. Everything should stay in the thiosulfate solution for at least 24 hours.

Keep the fish in quarantine in the chemical hood for 24 - 48 hours, and change the water two times within 24 hours. Inactivate the water in thiosulfate solution. Only then transfer the fish back to the system. (Within the fish, the mutagens are less stable than in water, due to the presence of reactive groups e.g. in proteins and DNA).

## DETAILED PROTOCOL

1. Take ENU (Sigma, 1g) in sealed/Isopac bottle out of can (stored at –20˚C) and equilibrate to room temperature for 15 min.

2. Put 3 kg sodiumthiosulfate in inactivation tank, dissolve in 12 l warm tap water. Adjust pH to 10 by adding NaOH solution (good for 30 liter final volume of inactivation bath, including mutagen solution, etc.) set up in fish tank, 40 liter, or similar.

3. Inject 85.4 ml 10mM $NaPO_4$ buffer (make by adding 5ml 0.5 M $NaPO_4$ buffer pH6.0 to 245 ml $H_2O$, final pH is 7.4). Two steps: 45 + 40.4 ml. Due to injection, air pressure in bottle will increase, let air escape only into syringe, not in environment i.e. after injection of buffer, move push-in part of syringe back to original location before removing syringe/needle from bottle. Remove air in syringe into inactivation tank)

Note: use 60 ml syringe, measure volume by looking at buffer/air border, not by looking at end of syringe. e.g. straight buffer/air border at 45 ml, syringe border at 50 ml. Inject 40 ml by pushing buffer/air border down to 5 ml. Hold the push-in part of the syringe such that no dripping out occurs.

4. Place ENU bottle in plastic box on a shaker. Fix the ENU/buffer bottles to box with tape, shake for 60-90 min at level 4.5 on shaker in hood. Before turning on the shaker, reduce speed to 0

5. Fill 2 l Erlenmayer flasks with 992 ml eggwater + 1 ml 0.5 M NaPO buffer pH 6.0. Leave alufoil on. Put in waterbath (12 l distilled water) and set the temperature to 26° C, if necessary by addition of ice.

6. Put 6-8 male fish into each Erlenmayer. With some skills you can catch three fish in net, close your fingers around them, and put them in Erlenmeyer together..

In the following steps ENU solutions are in direct contact with the environment. Be even more extremely careful than before!!

7. Put dissolved ENU solutions (30ml for 3mM final concentration) into Erlenmayer as follows: Inject 50 ml of air into bottle and suck off 50 ml ENU solution. Adjust straight buffer/air border. Inject 30 ml into each Erlenmeyer. Hold push-in part of syringe such that no dripping occurs!!!!! Use same strategy for rest of ENU solutions.

8. Check time now for start of mutagenesis

9. Inactivate the remaining ENU in bottle by injecting 90 ml of inactivation solution. Dump bottles in inactivation tank.

10. Incubate fish for 1 hour in ENU solution at 26° C, turn lights off, close hood. Check temperature 1-2 times.

11. Pour Erlenmayer content through net into inactivation tank. Put fish into fresh water cage (15/cage). This is the time fish may die, maybe because of stress and heart attacks. Expose fish to as little harsh treatment as possible (including catching, putting in tank, and noise e.g. by touching tank). Put each Erlenmayer flask into inactivation solution such that it is filled with this solution.

12. Leave fish alone until next morning. I would not fish out seemingly dead fish (belly up) until late in the evening, because 1) some recover, and 2) fishing out stresses still living colleagues. Before you leave in the evening, check water level in waterbath.

13. Morning: Put fish in cages with fresh water. Discard cages and water in inactivation tank.

14. Noon: Feed shrimp.

15. Late Afternoon: same as morning.

16. After 1 h you can put fish in system again.

This mutagenesis is repeated three times at 2-3 days intervals with the same fish to obtain optimal levels of mutagenesis in Medaka.

## Set up of F1 and F2 families (3 weeks+3 month+2 weeks+3 month)

Four weeks after the ENU mutagenesis, mutagenized G0 males are mated with wild-type females to generate F1 progenies. Grow up about 80 F1 progenies per G0 (F1 family). After obtaining the F1 progenies, mate G0 male with the tester strain female to carry out a specific locus test.

When F1 fish become fertile start to set up single pair crosses from each F1 family and grow up 60 fish/pair in a tank (F2 families). We usually make about 30 F2 families/F1 family.

## Screening

Nine-Ten months after the ENU mutagenesis, one can now start to do screen for mutations.

## Day1

Set up to eight single pair crosses/F2 family to recover mutations in one F2 family. It is recommended to keep the rest of the fish in the F2 family as a back-up stock.

## Day2

Collect eggs of successful matings on three successive days. Rolling clutches in the petri dish separate eggs by tearing the attachment filaments off the chorion.

Incubate eggs from 6 pairs in one six-well tissue culture plates at 28 C in 1x hatching medium and subject them to screening.

Score phenotypes on three successive days. Take pictures of mutants if necessary.

## Day3,

after 24h (approximately stage 18), inspect the neurula stage embryos for abnormalities in the brain, somites and the notochord..

## Day4,

after 48h (approximately stage 25), examine the early organogenesis stage embryos for formation of lens, optic cup and compartmentalization of the brain.

## References


1. Egami, N, Hyodo-Taguchi, Y. An autoradiographic examination of rate of spermatogenesis at different temperatures in the fish, *Oryzias latipes*. Exp. Cell Res. 1967, 47, 665-667.
2. Solnica-Krezel, L, Schier, AF, and Driever, W: Efficient recovery of ENU-induced mutations from the zebrafish germline. Genetics 1994,136:1401-20.
3. Driever, W, Stemple, D, Schier, A, and Solnica-Krezel, L: Zebrafish: genetic tools for studying vertebrate development. Trends Genet. 1994, 10:152-9. (a comprehensive review for mutagensis screen)
4. Shima, A & Shimada. A. :Proc. Natl. Acad. Sci. USA, 1991, 88: 2545-2549.
5. Loosli. F, Köster. RW, Carl, M. Kühnlein R, Henrich, T, Mücke, M, Krone, A, Wittbrodt, J. A genetic screen for mutations affecting embryonic development in medaka fish (Oryzias latipes), Mech. Dev. 2000, 97, 133-139.

## 2.2 Freezing medaka sperm samples

(Annette Krone and Jochen Wittbrodt)

Cryoconservation of sperm is a very suitable strategy for the preservation of important strains or mutants. This procedure is routinely used in aqua culture for a variety of different species (Rana and Mc Andrew, 1989, Steyn and van Vuren, 1987, Wheeler and Thorgaard, 1991). Poorly defined media like milk powder, egg extracts or fetal calf serum used as cryoprotectants (Aoki et al., 1997, Chao et al., 1987, Wheeler and Thorgaard, 1991) may be the cause for variable fertilization rates. Here we describe a rapid and reliable protocol that uses sucrose and DMSO as cryoprotectants (Holtz, 1993). The protocol allows to efficiently freeze and recover sperm obtained from living fish.

In brief sperm is obtained from the males by gently squeezing their testis with a pair of millipore forceps. The sperm is subsequently mixed with freezing medium in a glass capillary. Sperm containing capillaries are brought to successively lower temperatures and are eventually stored in liquid nitrogen. Successful recovery of the preserved stock after several month of storage in liquid nitrogen is routinely achieved by in vitro fertilization.

### Material needed:

- Triacine (Sigma# A-5040)
- DMSO (Sigma# D-8418)
- Blunt forceps (millipore filter forceps cat # XX62 000 06)
- Glass capillaries (Desaga GmbH Heidelberg, Postfach 101969, D 69009 Heidelberg, Cat # 12 01 96, the dimensions are 32 mm x 0.9 mm outer diameter)
- Cryo-tube: Nalgene cryogenic vials 5ml cat # 5000-0050
- 15 ml Falcon polypropylene tubes
- Sharp forceps (Dumont No 5 Biology)
- Pair of dissection scissors
- Ruler
- Timer
- Dissecting scope
- Ice bucket with dry ice
- Small Dewar with liquid nitrogen
- Plastic teaspoon
- Depression slides

## Buffers and solutions:

### Triacine/MS222 Stock solution:

| | |
|---|---|
| Triacine | 400 mg |
| $H_2O$ | 97.9 ml |
| 1M Tris pH9 | 2.1 ml |

pH should be 7.0

Aliquots of stock soln are frozen and kept at -20°C

To anesthetize fish freshly mix 4.2 ml of the MS 222 sock and 100 ml tank water.

### Ginzburgs Fishringer

| | |
|---|---|
| NaCl | 6.5 g |
| KCl | 0.25 g |
| $CaCl_2$ | 0.3 g |

add 800 ml $H_2O$

freshly add 0.2g $NaHCO_3$ and fill up to 1000ml with $H_2O$

### Freezing medium

| | |
|---|---|
| Sucrose | 0.6 M |
| DMSO | 10 % |

freshly prepared

as stocks dissolve 20.54 g Sucrose in 100 ml $H_2O$ and freeze 900µl aliquots

To set up the freezing medium (always freshly prepared, briefly stored cold in the dark) mix 900µl Sucrose stock and 100µl DMSO.

## Protocol

1. Isolate male fish one day prior to the sperm squeezing.
2. Prepare dry ice, freezing tubes, ruler and all the material you will need. For successful sperm freezing you will have to work quickly!
3. Prepare MS222 mix to anesthetize the male fish
4. Prepare the freezing medium and store cold in the dark
5. Anesthetize the male fish for 5 to max. 12 min.
6. in the meantime put 50µl of freezing medium onto a depression slide
7. Catch the fish with a teaspoon and put it into a foam bed, the belly up. Keep the fish wet with Ginzburgs fish ringer.
8. Under the dissecting scope remove traces of feces with a tissue paper.
9. Use a pair of millipore forceps to carefully squeeze the sperm out of the fish. Use the forceps to stripe the sperm out of the testis beginning in the middle of the belly close to the air bladder. Stripe carefully towards the anus. Suck up the sperm (opaque) into a microcapillary.

10. work fast now!!!
11. put the fish back into fresh tank water.
12. measure the level of sperm in the capillary with the ruler (e. g. 2 mm) and make a mark on the capillary at 3x sperm volume (e.g. 6 mm)
13. suck up freezing medium to the mark (6 mm). mixing occurs during this procedure. Extra mixing will only introduce air bubbles and lead to loss of sperm!!
14. put the capillary into the prelabelled cryo-tube. Transfer the tube and capillary to a 15 ml falcon tube that has been precooled on dry ice. Freeze there for 20 min.
15. Screw the cap onto the cryo tube containing the sperm capillary and put it into liquid nitrogen. Avoid to warm up the tube!
16. once thawed, the sperm can not be refrozen!!!!

---

## 2.3 In vitro fertilization of medaka oocytes with frozen sperm

### Solutions

#### 1x Yamamoto Ringer

| | |
|---|---|
| NaCl | 1.00 g |
| KCl | 0.03 g |
| $CaCl_2 \times 2H_2O$ | 0.04 g |
| $MgCl_2 \times 6H_2O$ | 0.10 g |
| $NaHCO_3$ | 0.20 g |

dissolve in 800 ml $H_2O$, adjust pH to 7.3, fill up to 1000 ml and sterile filter.

#### Hatching solution

| | |
|---|---|
| NaCl | 1.00 g |
| KCl | 0.03 g |
| $CaCl_2 \times 2H_2O$ | 0.04 g |
| $MgSO_4 \times 7H_2O$ | 0.16 g |
| Methylene blue | 0.0001 g |

dissolve in 800 ml $H_2O$ and fill up to 1000 ml.

### Protocol

have prepared one day in advance

◊ isolate females that produce many good eggs. Keep them single and prevent any stimulation by males or other females to prevent them from spawning.

prepare on the ivf day:

◊ get the sperm frozen in liquid nitrogen in a small dewar

◊ get some fertilized eggs for a positive control

### Preparation of the donor female

1. kill the female by decapitation with a pair of scissors
2. remove the ovary and isolate ripe oocytes
3. transfer the oocytes into a petri dish with 1x Yamamoto ringer and submerge carefully
4. remove the follicle layer with a pair of sharp forceps, keep oocytes submerged

### In vitro fertilization

5. add 100 µl 1x Yamamoto ringer into a depression slide
6. rapidly thaw one sperm capillary between the fingers and transfer the sperm to the ringer. Mix carefully and rinse the capillary.
7. remove as much as possible ringer from the oocytes and overlay them with the sperm suspension. Incubate for 2 - 5 minutes.
8. monitor the fertilization (after the incubation) under the dissecting scope. Cortical reaction (concentration of oil droplets at the vegetal pole) will start in fertilized eggs. Unfertilized oocytes will stay opaque.
9. transfer fertilized eggs into a petri dish with hatching solution and incubate at 28°C

References:


Aoki, K., Okamoto, M., Tatsumi, K. and Y. Ishikawa (1997) Zoological Science, 14: 641-644.

Chao, N. H., Chao, W. C., Liu, K. C. and I. C. Liao (1987) J. Fish Biol., 30: 107-118.

Holtz, W. (1993) *Aquaculture,* **110**: 97-100.

Kirchen, R. V. and W. R. West (1976) In: The Japanese Medaka: Its care and development. Carolina Biological Supply Co., Burlington, North Carolina.

Rana, K. J. and B. J. Mc Andrew (1989) *Aquaculture,* **76**: 335-345.

Steyn, G. J. and J. H. J. van Vuren (1987) *Aquaculture,* **63**: 187-193.

Wheeler, P. A. and G. H. Thorgaard (1991) *Aquaculture,* **63**: 35-44.

Yamamoto, T. (1975) In: Medaka (Killifish), Biology and Strains. Keigaku Publishing Company, Tokyo.


---

## 2.4 Freezing zebrafish sperm samples

(J. Belak, J. Gruenbaum, Z. Rangini, W. Driever)

The protocol for cryopreservation of sperm was modified from the one developed in the Eugene zebrafish labs (Westerfield, 1994; "http://zfish.uoregon.edu/zf_info/zfbook/zfbk.html")

The method was changed to use standard plastic cryovials (see below), and times for freezing adjusted accordingly.

Freezing medium was prepared as follows: 9.0 ml Ginzburg Ringer (6.5 g NaCl, 0.25 g KCl, 0.3 g $CaCl_2$, 0.2 g $NaHCO_3$ per liter of $H_2O$; pH adjusted to 8.0), and 1.0 ml methanol were mixed, and then added at room temperature to 1.5 g Carnation nonfat milk powder. The solution was then thoroughly

mixed by shaking for 5 minutes on a vortexer. Male adult zebrafish were anesthetized with Tricaine (3-amino benzoic acid ethylester; 168 mg / liter, pH 7.2; Sigma) and rinsed in egg water. The male was placed ventral side up in a slit cut into a polyfoam sponge. Excess water was blotted off with paper towel and 0.25 to 2.00 µl of sperm were collected from a male into a 1-5 µl calibrated glass capillary, gently squeezing the male with Millipore filter forceps. The sperm was added to 10 µl of prechilled (on ice) freezing medium and immediately mixed in a prechilled (ice) microcentrifuge tube. The resulting mixture was drawn into the capillary, which was rapidly transferred to a 5.0 ml Falcon cryovial, placed into a 50 mL Falcon polypropylene centrifuge tube. Both the 5 mL and 50 mL tube had been prechilled on dry ice. The freezing assembly was submerged horizontally in dry ice for 30 minutes. Finally, the 5.0 mL cryovial with the sample were transferred quickly to liquid nitrogen for storage.

We keep track of frozen samples using a File Maker Pro database. We record: genotype of frozen sperm sample; stock number of the male, date of freezing, amount of frozen sperm within the sample, quality of frozen sperm sample (a measure for density of sperm suspension: "good";= milky white, "OK" = milky, but transparent; "not good"= diluted appearance; however, even samples classified as "not good" frequently give good numbers of fertilized eggs, given that large egg clutches are used.

---

## 2.5 Fertilization with frozen zebrafish sperm samples

(J. Belak, J. Gruenbaum, A. Fainsod, Z. Rangini, W. Driever)

The protocol for in vitro fertilization with cryopreserved sperm was modified from the one developed in Eugene zebrafish labs (Westerfield, 1994; http://zfish.uoregon.edu/zf_info/zfbook/zfbk.html

The major differences to the previously published method is the use of a different buffer for in vitro fertilization, which was optimized in Abraham Fainsod's lab.

Female zebrafish from six to eleven months of age were maintained in tanks separate from males. To induce oocyte maturation, females were set up for crosses in breeding traps, two females and one male, in the late afternoon. The following morning, immediately after the lights were turned on, males and females were separated. Within the next two hours, in vitro fertilizations were performed. Females were anesthetized with Tricaine, rinsed with egg water, blotted dry on a paper towel, and transferred to a 6 cm petri dish. Eggs were expelled from the females by applying gentle pressure with dampened finger tips to both sides of the abdomen. Eggs were removed from the body of the female with a very fine (0.1 inch) paintbrush, and the female was transferred to a recovery tank. If at least 200 good eggs (of clear, slightly yellowish appearance) were obtained, they were used for in vitro fertilization.

70 µl of ice cold freshly prepared "I-buffer" (116 mM NaCl, 23 mM KCl, 6 mM $CaCl_2$ , then add 2 mM $MgSO_4$ 29 mM $NaHCO_3$ and 0.5% fructose; pH to 7.2 and filtered through 0.22 micron filter) was placed next to the eggs in the petri dish. The cryopreserved sample was removed from liquid nitrogen, rapidly thawed, and quickly mixed with the drop of ice cold I-buffer, to dilute the cytotoxic methanol.

Immediately, the sperm sample in I- buffer was mixed with the egg clutch by tilting the petri dish, and incubated for 15 to 30 seconds. Fertilization was activated upon addition of 750 µl of 0.5% fructose in egg water. After 2 minutes more 0.5% fructose in egg water was added to fill the dish.

AB strain or AB-Tü hybrid females aged 6 to 11 months can be used for in vitro fertilization every three to four weeks. Egg clutches of 300 - 400 eggs can be obtained routinely from most of the females, and fertilization rates with frozen sperm samples are usually 30 to 60%. In less than 10 %

of the samples, no eggs are fertilized. Therefore, if three or more sperm samples of a genotype have been frozen, the mutation can be safely recovered.

---

# 2.6 Identification of Sts Markers Closely Linked to A Mutation in medaka

Contributed by K. Naruse, T. Jindo, T. Kitagawa, H, Takeda, H. Mitani and A. Shima

The identification of linkage between the mutated locus and polymorphic marker is the first step to identify the genes that underlie mutant phenotypes. The presence of two genetically distinct, highly polymorphic popµlations (northern and southern) and their inbred strains makes medaka a good tool for such studies. Sequence comparisons of orthologous loci showed that single base-pair polymorphisms between these two strains occur at about 3% in introns and 1% in exons. Sequence Tagged Site (STS) markers, M makers are selected from 24 medaka linkage groups, and their polymorphisms coµld be identified with their PCR products size without a restriction-enzyme digestion. They can facilitate the mapping procedures using medaka.

## Sexual cross for mapping panel

1. Heterozygous mutant carrier fish (derived from southern popµlation) are crossed with HNI or Kaga strains (derived from northern popµlation) .
2. Identify the fish which are heterozygous for mutant allele and wild type allele derived from HNI or Kaga strains.
3. Cross such heterozygous carrier fishes with each other and collect at least 30 mutants and 30 wild type sibling fishes. If the mutant is homozygous viable, you can use backcross.

## Genomic DNA extraction

There are several genomic DNA extraction methods. DNA shoµld be extracted from each individual. To examine large number of samples 96 well format DNA extraction or automatic DNA extraction methods are recommended. This protocol is one of the examples for the small scale DNA extraction method.

1. Wash each egg with regent grad water and put each egg into 1.5 ml tube.
2. Freeze egg at -80°C
3. Add 200 µl of TNES-6U buffer (10mM Tris-HCl, pH 7.5; 125mM NaCl; 10mM EDTA; 1% SDS; 6M Urea) and homogenize with a plastic pestle.
4. Add 5 µl of protenase K solution (20 mg/ml) and incubate at 37 °C over night.

5. Add 300 □l of phenol/chloroform solution (TE saturated phenol : chloroform : Isoamyl Alcohol = 25 : 24 : 1) and mix well for 10 minutes by inversion.
6. Centrifuge at 10.000 rpm at room temperature.
7. Transfer aqueous phase to a new tube and add 500 □l of diethyl ether, mix by inversion.
8. Centrifuge at 5000 rpm for 2 minutes and remove ether and lipid phase.
9. Add 20□l of 5M NaCl and 500 □l of 100% cold Ethanol (-20 °C) to the aqueous phase and incubate at -80°C for 1 hour.
10. Centrifuge at 15000 rpm for 15 minutes at 4°C.
11. Remove supernatant and add 1000□l of cold 70% Ethanol (-20 °C).
12. Centrifuge at 15,000rpm for 10 minutes at 4°C and remove supernatant.
13. After drying add 250 □l of TE solution.

## Identification of the linkage group linked to a mutation using the bulk segregant analysis with M markers

1. Mix 2 µl of each DNA solution extracted from 30 mutant and 30 wild-type fish in each 1.5 ml tube. You must use the sibling wild type and mutant fish.
2. Wells at column number 1 to 6 of PCR plate are used for mutant DNA and wells at column number 7 to 12 are used for wild type DNA. . Mark left half of plates as “mutant” and right half as “wild” (see Fig.1).
3. Dispense 2 µl of primers of M markers( 48 markers for 24 linkage groups) to two wells for mutant DNA and wild type DNA.
4. Prepare 2 tubes of 850 µl of PCR master mix.
5. Add 50 µl of pooled DNA from mutant or wild-type to PCR master mix.
6. Dispense 18 µl of PCR master mix with mutant DNA to each well of column number 1 to 6.
7. Dispense 18 µl of PCR master mix with wild type DNA to each well of column number 7 to 12.
8. Cover PCR plate with silicon rubber.
9. Centrifuge PCR plate at 500 rpm for 1 minute to spin down the solution and mix by voltexing.
10. Run thermocycling.

### PCR master Mix

| | |
|---|---|
| Taq Buffer (10 X) | 100 □l |
| dNTP (10 mM dNTP) | 5 □l |
| DDW | 738.5 □l |
| Taq polymerase (Qiagen or Takara EX) | 6.5 □l |
| Total volume | 850 □l |

### PCR condition

1 cycle 95 C - 120 sec.
30 cycles 95 C - 30 sec., 55 C - 30 sec., 72 C - 60 or 180 sec.
1 cycle 72 C - 300 sec.
4 C -for keeping

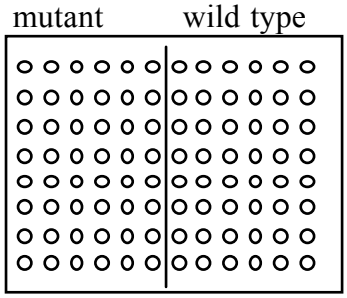



Fig. 1. 96 well PCR

## The primer sequence of M markers

| LG | Gene(EST) | Forward Primer | Reverse Primer |
|---|---|---|---|
| LG1 | SL1 | CCTGCAATGGGAAATTATTCTGCTC | CTTTTGTGTCTTTGGTTATGAAACGATG |
| | HT32075 | TGTCATTGCATCAGGTCATA | CCCCGGAATTACTCGTCTCT |
| LG2 | HT33063 | AAGTTCGTTCATTCGCACAC | CAGAGGAGGAGGAGGACGAT |
| | OLa0102f | CGGCTTCACCAGACCTCACCT | CGCAGGATCTTCAGTTTCTCGTCCA |
| LG3 | HT33005 | CGAAACATGCCGCTAAAGGT | GCCGGCGCTCCAAAGATGTG |
| | SC3ctg48 | CATATCTCATTTTTAATGCCGTCAC | AGAATTTGGAGGTTGGTTGCT |
| LG4 | OLa0808d | ATCTTCAGTCTTTGGCTCCGGCC | TCCCTGTTGGAGAAGCGGACAAT |
| | OLb1902c | GAATCCAGACATGGCCATCCGATATC | TCTTCCTCTTGGCCCGAATGTGG |
| LG5 | HT32067 | GAATCTCACTTGCAGGGTTT | TCACAAAACATGCCCTAACC |
| | OLc0203a | TCTTCTTCCACACCGATGCCGC | AACAAGTGCCAGAACGAAGCCCC |
| LG6 | OLb1207b | CTGCTGGGGTAACTGCTGCCTCACA | TGGTTTGGCTCCAAGGTTGGGAT |
| | HT133079 | CGGCGGCGTGTTCGGGTCAG | GACGGTTCCCCTCTTGCTCT |
| LG7 | SC3ctg41 | TTTTTGGATGATGACGTTTGTGCAA | TTCAGTCGCAATGCATTGTGCTCTA |
| | OLa2810d | GCCTTCAGTGTGTGAGCTCAGATTTCTG | CATGATGTCTGCGCTGTTCCCG |
| LG8 | OLb1911b | GCTGTCGGAAGGTTTTATTTC | AAGAGGTCGTAGATGTAGCGA |
| | OLa0405c | CTCTCCCCCTCTTTGAGAAGA | CCAAAACCTGCAGAGAAGCTG |
| LG9 | HT151087 | CTGGGTCCCATTGTAAACTT | GCGTGCTGGTGGTGGGTTAC |
| | OLb0711c | CAAGAAACTGTGCGACCTAGAACCG | TGCAACGACACTTCCTGTGACACAC |
| LG10 | HT152001 | ATTTAGAAAACCGCTTGTGT | CACTGTGGTGAAGACTGTTG |
| | OLa0407e | TCGTGCAGCCTCTGAGTCGGTGTT | CGACTGAATCAGCTTCTCCCCATCT |
| LG11 | UBA | TCCATCAGTGTCTCTCCTCCAGAAG | GCAGACACAGAGAGAATGACAGCG |
| | OLb2808a | GATGAATTTCTGGGAGAACGGACCA | GATGCCCCCAAAATGGTGTTATCAT |
| LG12 | OLb2408a | TCTGGCTAAGAACCCTGACCAAAGG | GCAGGAGTCTCCAACCAGATCACTG |
| | Olgc1 | GGCCGAGCCGTGACTTTATTTGTTCTAG | GAGGGTGCTTTGCTCCACAGTTACACAA |
| LG13 | HT181023 | GATCATAACAGCAGAGAACCAACATG | TCTGTTGTGTGCGTTCCAGCCGGTC |
| | SC3ctg58 | TTTTCTAGAACACCGAGCACCAGCG | TTTTACGCAGCACACAGACTCATGC |
| LG14 | HT182015 | CACCTATTCCAAACCTCGTCTT | CGATTATGCTTCCTTTGCCGAT |
| | OLa0301h | TTGCCTCTGTTCTTGTTTCGTGTGC | GGGCTTATATCCACCATGACGGTCA |
| LG15 | dd039 | TGGGAGTGGTGTAGGACTTCTT | TGTTAAACGAAGGGGCTTGTTAG |
| | OLb0905e | ACGTATTGTCAAAAGAGTTAAGGTG | GGGAAAATTACCCCAACTTAAAGAT |
| LG16 | OLb0505h | TTGGTCTGAGAAGGTTTGCAGATGC | AAACTGGCCCACGGTCGAATG |
| | Olgc2 | CAGCAGAGGGTCGTAAGAGG | CGTCTTTATTTATTCACTCCTTCAG |
| LG17 | OLb1505d | AAACAGACCCCTTTAATGACGAGCG | GCATCGGAGAATCATCTGGTGGAA |
| | Phr | GAYAAYTTYTGCTTCTACAAC | CATGGANGCCYCAGATNGACCACAT |
| LG18 | HT151011 | CAACCCATCTTACCTACTGAG | ATTACTGACAACAAGGGACTGA |
| | DAB | GTCTGGAGAGAAAATCTCCTG | TCTGACTCTGGCATGGACGGGT |
| LG19 | HT181001 | TAACTGGGTAAAATAAGAGCTGTGGTGC | GTAAGGTTTCATTCAGACACAAGG |
| | OLb1502f | CAGATCATGGCGTGTGTGTTGGC | TCGGGATCCTGTTGACGCTCA |
| LG20 | OLa1204c | ATCGGTCTTCCAGCGGACTTGTTC | TTTCCCCTCTCCGCATGAAAGC |
| | OLb1011h | TTTTTCACTAAGCACCAACTG | TGAACATCCACAGACTCGATG |
| LG21 | OLb1611a | CAGCTGAAAACATCTGCAGGATGCA | CACATGGAAACCTATCTGTCGCGC |
| | OLb1702c | CCAAAAACGCTCCAATTTAGCAACA | GAAAAAACTTCTGCACCGCCTCTG |
| LG22 | HT181069 | CCTGTCCCTGTCTCTGGTGG | CCCTGTCATGCTGCCCGATTA |
| | OLb0805g | AAGTCATGTCAGGGTTTCTGGACGG | CACCTGGCCATTTGAAACTGCATTT |
| LG23 | B2m | ATGAAAGAGCTTTTCTTCATTGC | CTGGCCAGGGTCATGACTGTACAC |
| | OLe1106c | AAGAAGAANGGATCCAAAACTCAAACCG | TTCAATCATGGTCATGCCGGGTTT |

| | | | |
|---|---|---|---|
| LG24 | OLa0305a | CCTGAAGACTAAAGCTGCTCCAGCA | TGCCCTTGCCCTGCAGTTAAGAC |
| | OLb0311h | ACAGACACATAATTGCTCTCCGGCA | GAGGGAACGAAGCTTTCACCAGTTC |

## Separation of PCR products (Native PAGE)

1. Prepare 32 ml of separation gel mixture for 4 mini-gels.
2. After polymerization of separation gel, add 3 ml of stacking gel mixture.
3. A 4.5 mm pitch 18 well sample comb is used for making sample slot.
4. Irradiate fluorescent light to polymerize stacking gel.
5. Assemble electrophoresis apparatus
6. Fill the buffer chamber with Tris-Glycine buffer (0.0625 M Tris-0.048 M Glycine, pH8.4: 1/4 dilution from original protocol)
7. Run gel at 300 V for 50 minutes.
8. Stain gel with ethidium bromide for 5 minutes.
9. Put gel onto a UV transiluminator for photographing (prefer shortwave UV (254nm) transiluminator because of high sensitivity).

## Determination of the candidate linkage group of mutation

1. Compare the PCR band patterns obtained from wild and mutant DNA as templates.
2. Identify M markers showing the different band pattern between wild type and mutant. If the mutant locus linked with one M marker, it shows 2 bands in wild type and 1 band in mutant.

M LG21 LG22 LG23 LG2

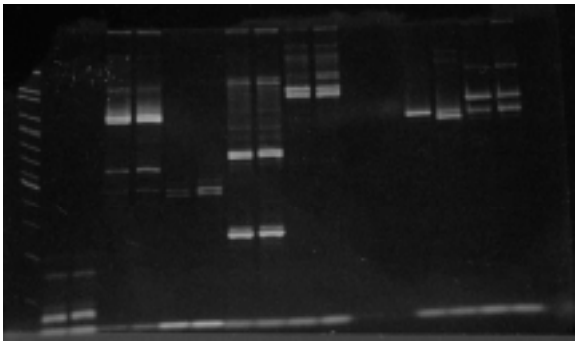


Fig. 2. An example of bulk segregant analysis.
Electrophoretic patterns of OLa0305a (LG24) are different between wild type and mutant.

## Segregation analysis to determine the linkage group of mutation

1. Dispense 2 µl of the genomic DNA from each individual into each well.
2. Add 18 µl of PCR master mix with appropriate primers
3. Cover PCR plate with silicon rubber
4. Centrifuge PCR plate at 500 rpm for 1 minute to spin down the solution
5. Run thermocycling..
6. Run gels using the same condition in balk segregation analysis.
7. Identify the maker most closely linkage to the mutation using linkage analysis program like MAPMAKER

## Material needed

- Centrifuge for 96 well PCR plate
- PCR machine
- 8-channel pipette (for 1~10 □l volume)

- Polyacrylamide electrophoresis apparatus.
- 4.5 mm pitch 18 well (or 17 well) sample comb
- Power supply

## Reagents

- Acrylamide
- Bisacrylamide
- TRIS (HYDROXYMETHYL) AMINOMETHANE
- Glycine
- HCl
- Anmmonium persµlfate,
- Riboflavin
- TEMED
- M marker primer set
- Taq polymerase (Qiagen or Takara EX)
- dNTP
- Taq Buffer

## Stock solutions for polyacrylamide gel:

### Solution A

1N HCl 48 ml
Tris 36.6 g
TEMED 0.46 ml
Add DDW up to 100 ml

### Solution B

1N HCl 48 ml
Tris 5.98 g
TEMED 0.46 ml
Add DDW up to 100 ml

### Solution C

Acrylamide 30 g
Bisacrylamide 0.8 g

Add DDW up to 100 ml

### Solution D

Acrylamide 10 g
Bisacrylamide 2.5 g
Add DDW up to 100 ml

Solution G

Ammonium persμlfate 0.2 g
Add DDW up to 100 ml

Solution E

Riboflavin 4 mg
Add DDW up to 100 ml

## Gel preparation

| Separation gel | Stacking gel |
|---|---|
| A: 4 ml | B: 2 ml |
| C: 10 m | D: 5 ml |
| G: 16 ml | E: 2 ml |
| DDW: 2 ml | G: 2 ml |
| Total 32 ml | DDW: 5 ml |
| | Total 16 ml |

## References

Davis B. (1964) J. Ann N.Y. Acad. Sci. 121: 404

---

# 2.7 Fluorescence *In-situ* hybridization (FISH)

(contributed by Indarjit Nanda and Manfred Schartl, Wuerzburg)

**Objective:** To demonstrate fluorescence in situ hybridization of DNA probes to medaka chromosomes. Each participant will perform simultaneous two-color hybridization using two BAC probes.

**Probes**: BACs (From Wuerzburg)

Due to time constraints, participants will be provided with prepared chromosome slides of medaka. However, the method to obtain metaphase chromosomes will be briefly described during the course.

*In-situ* hybridization provides the most direct method to study the chromosomal localization of DNA sequences in cellular material fixed to a microscopic slide. The technique was first described more than 30 years ago (Pardue & Gall 1969), using radioisotopes to label nucleic acids and autoradiography to detect the hybrid sequences. The advent of recombinant technology in conjuction with the availability of new fluorochromes and more sensitive detection systems has contributed to the development of non-isotopic detection alternatives, such as fluorescence in-situ hybridization (FISH) that involve visualization of hybridized sequences at the target site through fluorescence microscopy. By now, FISH has become a popular experimental technique with immense applications in gene mapping and molecular cytogenetics.

Moreover, its application is given a high profile in the field of human genetics, where FISH is now performed routinely in virtually all diagnostic cytogenetic laboratories. FISH can also be a useful tool to help us understand the genome organization in lower vertebrates. Not only does it allow us to map unique or repetitive sequences onto chromosomes, it is most importantly useful in identifying chromosomal homologies (synteny) between poorly mapped and well mapped genomes. FISH can be informative in isolating quantitative trait loci (QTL) of importance in aquaculture or in other economically important organisms. Thus far the FISH technique is least exploited in fish cytogenetics and only a handful of published reports present localization of ribosomal and several repetitive DNAs. Detail protocols for FISH can be obtained from a number of practical books but they largely focus on human gene mapping. In this section we include a basic FISH working protocol solely addressed to fish chromosomes and will demonstrate the mapping of two different loci (caspase 6B and *SL1*) to medaka chromosomes through simultaneous two color FISH.

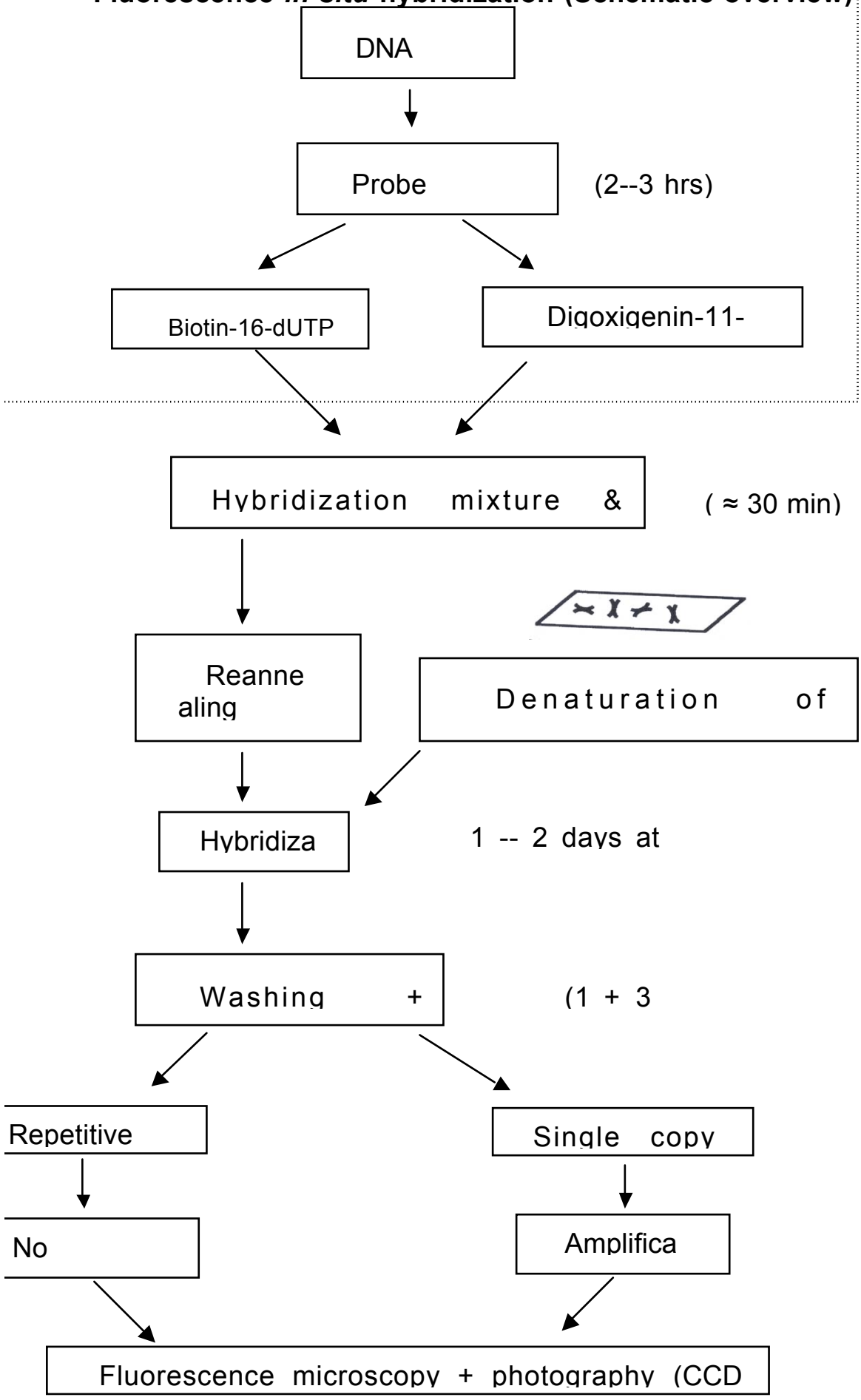
## Chromosome preparation

Conventionally fixed chromosome preparations and the biotinylated (or digoxigenin-labelled) probe mix are denatured separately and then hybridized together overnight (Figure). After washing, the hybridized probe is detected via FITC-conjugated avidin (or anti-digoxigenin). Therefore, high-quality cytogenetic preparations are important for optimum hybridization efficiency. Generally high quality chromosome preparation is feasible from *in-vitro* lymphocyte cultures following recent protocol of Fujiwara et al. (2001) from fishes in which blood can be easily obtained. Alternatively mitotic chromosomes can be obtained from moderately small-sized fishes directly from somatic cells using spleen and kidney. To arrest the metaphase cells, fishes are treated *in vivo* with colchicine (0.4 ml of 0.03% stock solution) for 4-6 hrs. The amount of colchicine and the length of treatment is dependent on bodyweight of the specimens. However, extremely small fishes like guppy and medaka are incubated over-night by adding colchicine (0.02%) to the water of aquarium.

1. put colchicine exposed fishes on ice, remove the organs after the animal has stopped movements for 30 sec.
2. release single cells by mincing the tissues in a small amount of hypotonic solution and then continue hypotonic treatment in 0.32% KCl solution for 40 minutes at room temperature.
3. collect the cells by centrifugation for 8 min at 1400 rpm and remove the hypotonic solution.
4. fix the material overnight at 4$^{o}$C in acetic acid/methanol (1:3 v/v).
5. wash and resuspend fixed chromosome preparations in a small amount of fresh fixative (0.5-1 ml), drop onto precleaned slide and flame dry.
6. control quality of preparation and yield of metaphases under microscope (phase contrast optics).
7.

Chromosome preparations are now ready for use in FISH experiments and can be stored as fixed solution at –20 $^{o}$C. Preferably 2 or 3 days old preparations are used in FISH experiments. The mitotic index and quality of metaphase chromosomes from direct *in-vivo* preparation are relatively low. Good quality mitotic chromosomes can be obtained from the fibroblast cultures prepared from fins.

**Fluorescence *in-situ* hybridization (Schematic overview)**



## Pretreatment of chromosome spreads (optional)

Remnants of cellular RNA and cytoplasm can impair the success of FISH experiments by hampering the penetration of probes to their target locus or due to unspecific interaction with the immunofluorescence detection system. These interferences can result in a low signal intensity and high background hybridization. In cases where low signal intensities are expected (e.g. small probe size) the conditions for FISH experiments can be optimized removing these remnants.

1. apply 300 µl of RNase A solution (100 µg/ml in 2xSSC) to slides and cover with coverslip.
2. incubate for 30 –60 min at 37°C in 2xSSC.
3. wash slides in 3x4 min in 2xSSC at room temperature.
4. incubate slides in pepsin solution (0.5µg/ml in 0.01 M HCl) for 5 to 10 min at 37°C and wash slides 3x4 min in 1x PBS.
5. place slides in PBS/50 mM $MgCl_2$ for 5 min.
6. fix in PBS/50 mM $MgCl_2$ / 1% formaldehyde for 10 min.
7. wash in 1xPBS 2x5 min (with agitation)
8. dehydrate the slides through an ethanol series and allow to air dry

## Probe labelling

Non-isotopic labelling of the DNA probe can be achieved through various methods such as nick translation, random primer labelling, PCR based labelling, end-labelling and *in vitro* translation. Many companies provide kits for labelling with ready made stock solutions with reliable and easy-to-follow protocols. Here we provide the protocol for labelling the probe through nick translation which is convenient and generally effective for large genomic fragments.

1. Mix the following in a 1.5 ml microfuge tube:
   - 2µg of whole probe DNA (free of RNA and phenol-chloroform)
   - 10 µl of 10x nicktranslation buffer (0.5M Tris/HCl pH 8.0; 50 mM $MgCl_2$; 0.5 mg/mL BSA)
   - 5 µl of ß-mercapto-ethanol (0.1 M)
   - 10 µl of unlabelled nucleotide mixture (0.5 mM dATP, dCTP, and dGTP)
   - 5 µl biotin-16-dUTP(1mM) or digoxigenin-11-dUTP
   - 5 µl of diluted DNase I (Roche): 1 µl of stock is diluted in 1 ml of distilled water (freshly prepared and keep on ice)
   - 2 µl of *E. coli* DNA polymerase I (20 units)
2. adjust to 100 µl with double–distilled water.
3. incubate the reaction for 90-120 minutes in a 15°C water bath.
4. stop the reaction by placing the tubes on ice.
5. check the size of the labelled products by running an aliquot (denature by boiling in a water bath ) on a 2% agarose gel at 120-150 volts using 100 bp ladder as a size marker.
6. if the labelled fragments are the correct size (smear range 100-400 bp), stop the reaction by adding 2 µl of 0.5 M EDTA and 1 µl of SDS (10%) and heat at 68°C for 10 min.
7. separate unincoporated nucleotides by spininng the reaction mixture through a Sephadex-G-50 spin column, equilibrated with column buffer (50 mM Tris/HCl pH 8.0; 1 mM EDTA, 0.1% SDS).

The fragment size of the labelled probe is critical for efficient hybridization to the target sequence. Probes larger than the optimum size give high back-ground signals and smaller-sized probe fragments may give weak signals. The amount of DNase I used here is optimum for large genomic probes (BACs, PACs). In case of smaller probes (e.g. cDNA) the duration as well as amount of DNase I may differ.

## Dot-blot assay to test incorporation of label

After labelling, it is necessary to check the incorporation of labelled nucleotides into the probe through a simple colorimetric assay using different dilutions of labelled probe.

1. spot 1 µl of the different concentrations of labelled DNA (e.g. 1/10,1/100) on a small piece of nitrocellulose filter (e.g 3x4 cm). Use 6xSSC for dilution of probe.
2. bake the filter for 30-60 min at 80°C.
3. soak the filter with AP 1 buffer (0.1 M Tris-HCl pH 7.5, 0.1 M NaCl and 2 mM $MgCl_2$) for 5-10 min at room temeperature in a dish.
4. discard the AP I buffer and incubate the filter in blocking buffer (0.1 g blocking agent (Roche) in 20 ml AP I buffer; will dissolve at 50°C) for 60 minutes at room temperature.
5. discard the blocking buffer, wash in AP1 buffer briefly and incubate the filter in avidin (or anti-digoxigenin) conjugated alkaline phosphatase (1:1000 dilution in AP I buffer) for 60 min at room temperature.
6. pour off antibody-AP mixture, wash twice for 5 min each in AP I buffer, then wash 3x4 min with AP II (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl and 50 mM $MgCl_2$) buffer.
7. discard the last washing buffer and cover the filter with color developing solution (45 µl of NBT and 35 µl of BCIP in 10 ml of AP II buffer). Incubate at least 1 hr in dark untill color develops sufficiently.
8. To stop the reaction wash the filter in TE buffer several times.
9. evaluate the signal intensity. At least the 10 pg spot should be clearly visible.

Prepare or purchase 75 mg/ml nitroblue tetrazolium (NBT) in 70% dimethylformamide and 50 mg/ml 5 bromo-4-chloro-3-indolyl phosphate (BCIP) in dimethylformamide.

## Hybridization procedure

Hybridization depends on the ability of denatured DNA strands to reanneal with their complementary target sequences. The details of hybridization parameters are extensively covered in several molecular biology text books. In the following the protocol is given for 10 µl hybridzation volume when a 18 $mm^2$ coverslip is used to cover the hybridization mixture on the slide. When larger areas are to be hybridized, scale up the solution accordingly. The majority of cloned genomic probes (>10 kb) contain interspersed repetitive sequences. Because of their ubiquitous nature, hybridization signals from repetitive sequences may obscure the specific signal from the unique sequences. It is therefore necessary to suppress such signals within the probe by incubating (before hybridization) labelled probe with unlabelled competitor DNA. Competitor DNA is either Cot1 DNA or sheared, sonicated genomic DNA (50-500 bp size). Standard hybridization is performed in 50% formamide, 2xSSC and 10 % dextransulfate and 100 ng-200 ng labelled DNA per slide (single copy) in presence of excess amount of carrier DNA. It is advisable to include calf thymus DNA as carrier DNA instead of using standard salmon sperm DNA for *in-situ* experiments with fish chromosomes.

### Probe preparation:

1. mix 100-200 ng of labelled DNA, 10 µg of carrier calf thymus DNA, 5 µg of yeast tRNA and 2-5 µg of competitor genomic DNA.

2. add 1/10$^{th}$ volume of 3M sodium acetate (pH 5.0) and 2.5 vol of 100% ethanol. Allow the probe to precipitate at least 2 hrs at –70$^{o}$C or ovenight.
3. spin for 15 min in a microfuge at 15000 rpm for 15 min at 4$^{o}$C and wash the pellet again with 70% chilled ethanol.
4. vaccum dry pellet and dissolve the pellet in 10 µl of hybridization mixture (50% formamide, 10% dextransulfate, 2xSSC, 5x Denhardt's solution).
5. vortex briefly an incubate at 37$^{o}$C for 30 min (to dissolve the probe).

For two-color hybridization co-precipitate both biotinylated and digoxigenin labeled probes.

## Denaturation of probe and chromosomes

For hybridization of chromosomal DNA and labeled probe it is necessary that both DNAs are single stranded. Since high temperature damages the chromosome preparation, they are denatured in 70% and hybridized in 50% formamide which reduces both denatuartion and hybridization temperatures

1. prewarm the slides to 50-60$^{o}$C in a hotplate in order to prevent sudden temperature changes when the slides are put into hot denaturation solution.
2. denature chromosome spreads for 30 sec–1.5 min in 70% formamide/2xSSC (pH 7.0) at 70$^{o}$C.
3. dehydrate the slides in a series of ice-cold ethanol (70%--- 100%) for 5 min each. Allow them to air dry.
4. denature the probe DNA by incubation at 75$^{o}$C for 10 min followed by chilling on ice.
5. if the competition of repetitive sequences is necessary, allow reannealing of probe with competitor DNA for 10min-1 hr at 37$^{o}$C, then put on ice.

Poorly fixed chromosomes have a tendency to over-denature and come off the slide during denaturation in formamide. Over-denatured chromosomes have a hollow, puffy appearance under the phase contrast microscope. Underdenatured chromosomes might show perfect morphology but have weak or no signal.

## Hybridization

1. Add 10 µl (or more) denatured probe mixture to denatured chromosome preparation.
2. put an 18 mm$^2$ (or higher range) coverslip on the probe mixture droplet. Avoid air bubbles.
3. seal the edges of the coverslip with rubber cement.
4. transfer the slides into a moist chamber and incubate at 37$^{o}$C for 1-2 days.

## Post hybridization washes

After hybridization, a series of washes is used to get rid of unbound probe. Increasing the washing temperature and / or decreasing the salt concentration will increase the stringency and remove unwanted background.

1. carefully remove coverslip and wash slides 3x5 min in 2xSSC and then transfer to a coplin jar containing prewarmed 50% formamide in 2xSSC (pH 7.0) at 42$^{o}$C in a shaking waterbath. Carry out washes in formamide solution for 3x5 min then three 5-min washes (with agitation) in 2xSSC at 42$^{o}$C.
2. transfer the slides to a new coplinjar containing prewarmed 1x (or 2x) SSC and continue washing at 60$^{o}$C for 2x5 min. After this step the slides are immediately processed for detection step.

## Detection

Detection systems for biotin-labeled probes usually employ the biotin-avidin affinity systems and those labelled with digoxigenin need anti-digoxigenin conjugated with a fluorochrome. For probes directly labeled with fluorochromes (special cases), no detection steps are required. After the detection step the slides are mounted in appropriate mountant and counterstain. Counterstaining allows visualisation of complete mitotic complement under fluorescence microscope and FISH signal on specific chromosome can be seen in the background of counterstained chromosomes. For fluorescent detection, most commonly FITC (blue excitation, yellow green fluorescence), rhodamine, Texas Red or Cy3 (all green excitation, red fluorescence) conjugates are used. If two or more probes are detected simultaneously, it is necessary to select the detecting systems, particularly when several antibodies and detection reagents are mixed and used simultaneously. In most cases single copy signal is enhanced through further rounds of staining with secondary antibody-fluorescence conjugates. Amplification can be carried out directly after detection of hybridization sites before counterstaining or after DAPI staining and examination under the microscope. However, amplification step also enhances the background signals. During the whole detection procedure the slides should not be left to dry.

1. after post hybridization washing, take the slides out of the coplin jar, drain them as efficiently as possible by absorbing solution from the slide edges with a paper towel and apply 300 µl of blocking solution (3% blocking reagent (Roche) in 4x SSC). Cover with a large coverslip (24x 60 mm) and incubate for 30 min at 37°C in a moist chamber. (Blocking agent needs to be dissolved at 60 °C with occasional vortexing).
2. allow the coverslip to slide off, drain excess fluid as possible and add 300 µl of detection cocktail (5 µg/ml avidin-rhodamine and FITC conjugated mouse anti-digoxigenin (1:100) in 4x SSC/0.1% Tween/ 1% blocking agent). Cover with a coverslip and incubate at 37$^0$C in a moist atmosphere for 30 minutes.
3. remove coverslip carefully and wash the slides in 4x SSC/0.1% Tween at 37°C for 3x5 min with gentle agitation. (**proceed for amplification, if required**)
4. wash the slides for 3x5 min each in 4x SSC/ 0.1% Tween (prewarmed) on a shaker and again wash in 2x SSC for 3x5 min and allow to air dry.
5. mount slides in Vectashield containing 1.0 µg/ml DAPI.

## Amplification protocol:

1. apply 300 µl of biotinylated anti-rhodamine (5 µg/ml in 4xSSC/0.1% Tween/1% blocking reagent), put on a coverslip and incubate in the dark for 30 min at 37°C in a moist chamber.
2. wash slides 3x5 min in 4x SSC/ 0.1% Tween at 37°C with gentle agitation.
3. apply 300 µl of avidin-rhodamine (5 µg/ml) and FITC conjugated sheep antibody against mouse (1:150 dilution) in a soluion containing 4x SSC/0.1% Tween/ 1% blocking agent. Cover with a coverslip and incubate in dark at 37°C.
4. wash the slides for 3x5 min each in 4x SSC/ 0.1% Tween (prewarmed) on a shaker and again wash in 2x SSC for 3x5 min and allow to air dry.
5. mount slides in Vectashield containing 1.0 µg/ml DAPI.

## Microscopy

Most modern epifluorescence microscopes are adequate for FISH analysis. The choice of filters and objectives needs to be carefully considered. Fluorescence microscopes usually contain fluorescence filter sets consisting of an excitation filter, a dichroic mirror and an emission filter that permit the sequential visualization of the various fluorophores for color photomicrography. If digital imaging system is not available, use 640 ASA color slide films for photography. Dual or triple bandpass filters allow

simultaneous visualization of differentially labeled probes and counterstain. However, the intensity of fluorescence is reduced with these filters. On the other hand CCD cameras have increased the sensitivity and flexibility of FISH techniques as it captures fluorescent signals not visible to normal human eye. The choice of counterstain depends on the fluorochromes used for FISH. The commonly used fluorochromes for counterstaining are DAPI and propidium iodide (PI). PI is not usually recommended as it fluoresces brightly over a wide range of wavelengths. Hence, in most cases DAPI is used as counterstaining. Both DAPI as well as images with specific FISH signals can be merged with FISH software. Single copy probe cloned in cosmids, YACs or BACs hybridize very efficiently so that only few metaphases need to be analysed. In contrast, the hybridization efficiency is greatly reduced for 2-5kb probes. With this in mind, in some situations it may be preferable to isolate a larger clone for mapping by screening BAC or PAC library.

## Evaluation

Since standard G or R-bands are difficult to induce on fish chromosomes a fluorescent signal on metaphase chromosomes obtained by FISH is specifically assigned by measuring its position relative to the end of the short arm of the chromosome (FLpter value). A disadvantage is that only low-resolution mapping is possible with highly condensed metaphase chromosomes since two markers need to be at least 1 Mb apart to be resolved as separate hybridization signals (Trask et al. 1991). However, in recent years higher resolution FISH became feasible on extended or mechanically stretched chromosomes (Heiskanen et al. 1996) that allow to distinguish markers that are 200-300 kb apart. Even markers that are less than 10 kb apart can be resolved using fiber-FISH (Michalet et al. 1997) but in this case chromosome morphology is lost.

## Material needed

### Probe labelling ( 2-4 hrs)

***Chemicals:***

- Biotin-16-dUTP (Roche; Cat No: 1093070)
- Digoxigenin –11-dUTP (Roche; Cat No. 1093088)
- DNase I, RNase free (Roche; Cat No: 776785)
- DNA Polymerase I (*E. Coli*) (NEB; Cat No: MO209S)
- Nucleotides (100 mM dATP, dGTP, dCTP ; GIBCO)
- 2-mercaptoethanol (SIGMA)
- Tris
- $MgCl_2$
- EDTA (0.5M pH 8.0)
- SDS (10 or 20%)
- Agarose

- Minigel (Complete set)
- Sephadex G50 DNA grade (autoclaved)
- Glass Wool (autoclaved)
- 1ml Syringes (OMNIFIX-F 1ml)
- 100 bp ladder (NEB 323-1S)
- $EtBr_2$
- Thermobath ( 15 $^oC$)
- microcentrifuge
- Waterbath (different temp.)
- Reaction tubes, microtips (sterile), micropipettes
- Pepsin (SIGMA, P6887)
- Formaldehyde 37% (MERK)
- 1N HCl (MERCK)
- Plastic petridisches (sterile), Disposable
- Transilluminator (gel documentation)

### *Test for probe labelling (optional)*

- Streptavidin-alkaline phosphatase (Roche; Cat No: 1093266)
- Anti-digoxigenin-alkaline phosphatase (Rohe; cat No: 1093274)
- Nitroblue tetrazolium chloride (NBT) (Roche; Cat No: 1383213)
- 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche; Cat No: 1383221)
- Nitrocellulose paper
- Oven to bake nitrocellulose paper
- Blocking agent ( Roche; Cat No: 1096176)
- 
- Duration: 2-3hr

### *Hybridization*

- Sonicated calf thymus DNA (10 mg/mL)
- E.coli t RNA (SIGMA, D-2001)
- Sheared-sonicated genomic DNA (medaka)
- Deionized Formamide (Appligene; FORMD002, 500 mL)
- 3M NaOAc, pH 5.2
- 100X Denhardt (Appligene DENH 1001- 5 mL)
- 50% Dextran Sulfate (Appligene DEXT 5002, 50 mL)
- 20x SSC (pH 7.0)

- Tween-20 Mol. Biol grade (Appligene Tween 201)
- Coverslip (24x60; 24x32; 21x26)
- Rubber Cement (Fixogum)
- PBS (10x) (Appligene PBS 10x02, 1 lit)
- Centrifuge (4$^{o}$C, 15000 rpm with rotor for 1.5 ml tubes)
- Table top Centrifuge (for 1.5 ml tubes)
- Speed Vac
- PH meter
- Waterbath ( 45 $^{o}$C; 60 $^{o}$C) with shaker
- Shaker (Standard: IKA-VIBRAX-VXR)
- Couplin Jars (Vertical)
- Fluorescein AVIDIN DN (Vector; A-3101)
- Rhodamine AVIDIN D (Vectror; A-2002)
- Biotinylated Anti-Avidin D (Vector BA-0300)
- Monoclonal Anti-digoxin-FITC conjugates (SIGMA F-3523)
- FITC conjugated Sheep Anti-Mouse IgG (SIGMA; F-3008)
- Moist Chambers (Horizontal, For incubation, Preferrably metal autoclave box)
- Vectashield Mounting Medium with DAPI (Vector; H-1200)
- Fluorescence Microscope (Epifluorescence, Neofluor objectives 20x, 100x)
- Equipped with CCD camera with appropriate software to deal with multiple
- Colors
- Filter Sets( narrow range): DAPI, FITC, Rhodamine
- Horizontal slide folder (Praeparate mappe)
- Deep-freezer (-80 c) (for precipitation of probes)
- Incubator (37 oC)
- Ethanol

## Buffers and solutions

### Nicktranslation buffer

| | |
|---|---|
| 0.5 M | Tris/HCl pH 8.0 |
| 50 mM | $MgCl_2$ |
| 0.5 mg/ml | BSA |

### Column buffer

| | |
|---|---|
| 50 mM | Tris/HCl pH 8.0 |
| 1 mM | EDTA |
| 0.1% | SDS |

### AP I buffer

0.1 M Tris/HCl pH 7.5
0.1 M NaCl
2 mM MgCl2

### AP II buffer

0.1 M Tris/HCl pH 9.5
0.1 M NaCl
50 mM MgCl2

### Hybridization mixture

50 % formamide
10 % dextransulfate
2x SSC
5x Denhardt's solution

### Blocking solution

3 % Blocking reagent (Roche) in 4x SSC

## Additional information

# A- Preparation of competitor DNA

1. prepare genomic DNA using standard procedure and dissolve in TE.
2. add 10 N NaOH to dissolved DNA so that the final concentration of NaOH should be 0.5 M.
3. heat at 96$^{o}$C for 15-20 min and then keep on ice (around 5-10 min).
4. add acetic acid so that the final molar concentration should be 0.5 M.
5. precipitate the DNA with iso-propanol and Na-acetate. Mix well and place on ice for at least 30 minutes.
6. spin down DNA, after a wash in 70% ethanol, vaccum dry and dissolve the pellet in TE.
7. check the length of the competitor DNA on a minigel which should appear as smear ranging between 100-500 bp

## B- Fluorescent dyes commonly used for FISH

| Fluorochromes | Color | Absorbance (max in nm) | Emission (max in nm) |
|---|---|---|---|
| DAPI (counterstain) | Blue | 356 | 461 |
| FITC | Green | 490 | 525 |
| Rhodamine | Red | 550 | 580 |
| Cy3 | Red | 575 | 605 |
| Texas Red | Deep red | 595 | 620 |
| Cy5 | Far red | 640 | 705 |
| Propidium iodide (counterstain) | Red | 535 | 617 |

## References


1- Fugiwara A et al. Genetica **111**: 77-89 (2001)

2- Pardue ML; Gall J. Proc. Natl. Acad. Sci **63**: 378-383 (1969)

3- Heiskanen M. et al. Trends Genet **12**: 379-382 (1996)

4- Michalet X et al. Science **277**:1518-1523 (1997)

5- Trask BJ et al. Am. J. Hum. Genet.: **48**: 1-15 (1991)


## Additional literature


1- Human chromosomes: principle and techniques; 2nd edition by R.S. Verma and A. Babu (McGraw-Hill, Inc. 1994)

2- Lichter P & Cremer T: in Human Cytogenetics vol I, 2nd edition IRL Press, 1992 (Eds: Roony DE & Czepulkowski BH)

# 3. MICROSCOPIC OBSERVATIONS

(M. Westerfield, Zebrafish book)

Live zebrafish embryos can be viewed at low magnification by placing them in depression slides filled with 30% Danieau's. After 16 h of development they become spontaneously motile and can be anesthetized with MESAB (see "Recipes"). After 23 h of development, they begin to develop pigmentation which can interfere with some observations. To maintain optical transparency grow the embryos continuously, starting around 15 h, in 1- phenyl-2-thiourea (0.2 mM in 30% Danieau's).

For observations at accurately determined developmental stages, it is imperative to maintain the embryos at 28.5°C at all times. This can be accomplished by keeping the embryos in small petri dishes or covered beakers on a microscope slide warmer and by using a microscope stage warmer, or alternatively by maintaining the observation room at the appropriate temperature.

---

## 3.1 Removing embryos from their chorions

(Source: M. Westerfield, Zebrafish book)

Some observations of zebrafish development can be made directly through the chorion, however for most procedures it is better to remove the chorion. When raised in 30% Danieau's at 28.5°C, zebrafish develop normally outside of their chorions. Chorions can be easily removed with sharp forceps by gently making a tear in the chorion and turning it upside down so that the embryo falls out. To remove the chorions from young embryos (up to 15 h of development) care must be taken when opening the chorion since these embryos are easily damaged. Pre treatment with a dilute solution of pronase (2 mg/ml in 30% Danieau's, 1 min. to 3.5 min, RT) makes the chorions brittle and easier to remove. Be sure to rinse the pronase treated embryos thoroughly at least 3x to remove all the enzyme. Embryos usually fall out of their chorions with gentle swishing during the rinse. Note that pronase often contains nuclease and other unwanted enzymatic activities which can be removed by self-digestion for several hours at 37°C. Embryos removed from their chorions can be transferred from one container to another by gently sucking them up with a fire-polished Pasteur pipette. Small glass petri dishes (35 mm diameter) are adequate for holding small numbers of zebrafish embryos during the first few days of development. The embryos can be brought to the center of the dish for viewing by gently swirling the saline with a circular motion.

*Dechorionated embryos stick to plastic and dissolve, thus dechorionated embryos should always be handled in glass dishes, or plastic dishes coated with a thin layer of 1% agarose in 30% Danieau's.*

Agarose coated dishes should be filled with 30% Danieau's after hardening of the agarose, for storage in the refrigerator. (Eggs , again, tend to stick if the agarose is dried out and rehydrates during the addition of eggs / 30% Danieau's!).

We often include Penicillin / Streptomycin (1:50- to 1:100 from Gibco-BRL stock 100x) with the 30% Danieau's (Em) to avoid bacterial infections, and increase survival rates.

## 3.2 Viewing chambers

(Source: M. Westerfield)

For higher magnification viewing with a compound microscope, mount the embryos between cover slips. A simple, disposable viewing chamber can be made from two large cover slips (25x60 mm) spaced apart with 2 smaller cover slips (22x22 mm). Using "Crazy Glue" (cyanoacrylate), glue one small cover slip near each end of one of the larger slips. Position the smaller slips so that one edge is flush with the long side of the larger slip. Then glue another small slip on top of each of the first two. Transfer the embryo in a drop of saline to the space in the center of the large slip between the spacers and gently cover with the other large slip. If the embryos need to be anesthetized, transfer them first to a small petri dish with the MESAB solution and then into the viewing chamber. The chamber can be placed on a standard microscope stage by taping it to a glass slide or by using a holder with a recessed edge and a central hole. To remove embryos from the chamber, dip one corner with the space between the smaller and larger slips into a petri dish filled with 30% Danieau's and direct a stream of saline into the space between the cover slips on the top edge of the chamber with a Pasteur pipette. The embryos will wash out into the dish and can be picked up with a fire-polished Pasteur pipette.

In the course, we will use modified viewing chambers: We use a regular microscope slide. We fix 6x22 mm cover slip stripes at 1 cm distance to the middle of the slide using "crazy glue". For early stages, we use 3 layers of strips, so the embryos will not get squeezed. For 24 hrs and older, it is often easier to use 2 layers only, which squeezes the embryo a bit, but makes it easier to get the embryo / larvae into a good focal plane. We use 18x18 mm #1 cover slips. These smaller cover slips can easily be moved around a bit to reorient the embryo for viewing.

## 3.3 Methyl Cellulose mounting

(Source: R. Warga)

For a very little rigid mounting medium, use a low concentration of agarose (0.1%) or 2%, 2.5% or 3% methyl cellulose (Sigma M-0387). Methyl cellulose is especially good for orienting young embryos (less than 10 h old).

1. Apply a drop of 3% methyl cellulose to a depression slide.
2. Suck an embryo up into a Pasteur pipette and position it near the opening in the pipette.
3. Plunge the pipette into the methyl cellulose and gently expel the embryo with as little saline as possible. The saline around the embryo will be quickly absorbed into the methyl cellulose.
4. Use a fine loop of nylon fishing line to orient the embryo.
5. Place a drop of saline over the well to keep the preparation from drying out and getting too sticky.

6. To release the embryo, drop the entire slide into saline for a few minutes. The methyl cellulose will absorb the saline and become very soft so that the embryo can be teased free with the nylon loop.

---

## 3.4 Agar mounting

(Source: J. Eisen)

For more precise orientation of zebrafish embryos and to keep them from floating around during long term observations, embed them in agar.

1. Prepare a 1.2% solution of agar in 30% Danieau's by heating in a boiling water bath or by using a microwave oven. This solution remains liquid at 37- 40°C, but quickly hardens when cooled.
2. Keep a small amount of solution (1-5 ml) liquid in a test tube in a bench top test tube warmer or water bath.
3. Transfer an embryo in a minimum amount of 30% Danieau's into the test tube with a fire polished pipette.
4. Quickly suck up the embryo and transfer onto a glass slide and add several drops of liquid agar.
5. Let the agar harden and then using a pointed scalpel, cut the embryo out of the droplet in a little block of agar.
6. Pick up this block with forceps, orient it as precisely as you wish on a fresh glass slide and cover it with more liquid agar to hold it in position.
7. To remove an embryo from the agar, place several drops of saline on top of the agar.
8. Using a pointed scalpel, create a "V" shaped cut in the agar with the point of the "V" aiming towards the embryo's head.
9. Hold the tips of a pair of sharp forceps closed and plunge them into the agar between the point of the "V" cut and the head of the embryo.
10. Gently open the points of the forceps creating a crack in the agar which will run along the length of the embryo.
11. The embryo will then float out of the agar into the saline and can be picked up with a fire-polished Pasteur pipette.

1. Change water in fish-cross traps immediately after lights go on (you will get much cleaner eggs if you do this).
2. Within 30' or so of when eggs are laid, collect them and dechorionate them with pronase in 30% Danieau's. Be careful not to over digest eggs with the pronase. If you don't get many eggs don't waste your time with the rest of the experiment.
3. Reserve most of the embryos in a fresh agarose-coated 100 mm petri dish to use as hosts. Take would-be donor embryos and load onto ramp(s). No more than about 30 per ramp. From now on cleanliness with the embryos is very important, and dead embryos must be promptly removed. It is best not to keep more than approximately 30 embryos in a 100 mm agar-coated dish for extended (days) time.
4. Orient embryos so that ventral surface is facing up the slope of the ramp (do this right at the scope you are going to use for the yolk-poke).
5. Backfill a yolk-poke needle with appropriate dextran sol'n, and put needle in holder hooked up to air pressure station.
6. Inject embryo. Different experiments require different injection strategies. For dye filling, inject embryos by putting the needle into the ventral side of the yolk ball, bringing the tip all the way through the yolk until it is just below or within the cytoplasm.

7. Inject a small bolus of dye (make a ball approx. 1/10-1/20 diameter of embryo). If the needle was properly positioned most of the dye should diffuse up into the cytoplasm within minutes and the entire blastoderm should look distinctly pink (if rhodamine-dextran was injected).
8. Yolk pokes can be done up to about the 8-cell stage, although earlier is better. More uniform dye labeling in 4-16 cell embryos can be accomplished by delivering several smaller dye injections at evenly spaced intervals in the blastoderm.
9. Remove embryos from ramp, put into fresh agarose-bottomed dish and keep them in the dark. After an hour or so, pick out the unfertilized/abnormal embryos.

---

# 3.5 Microscopic observation of live medaka embryos (timelapse)

(contributed by Martina Rembold)

## Removal of chorion

Medaka embryos are covered with a 2-layered chorion that has a hard inner layer and a soft outer surface. To observe Medaka development under the microscope or stereomicroscope it is necessary to remove the chorion in a two step protease treatment employing Proteinase K and hatching enzyme. The latter is a protease, which is sereted from the hatching gland and dissolves the inner layer of the chorion (Yasumasu et al, 1994).

**Dechorionated embryos are very sensitve**, especially in earlier stages, when they expose most of the yolk, thus:.

- They may not contact air, or air bubbles in the pipet, otherwise they will immediately collapse.
- Use either non-adherent plastic suspension culture dishes or Petri dishes covered with 1% agarose in BSS to prevent embryos from adhering to the plastic.
- Use wide mouthed pipets to prevent damage of the embryos (eg. Cell saver tips)
- Use sterile solutions and BSS containing antibiotics (penicillin and streptomycin) if you want to culture them for a longer period.

### Preparation of hatching enzyme (500 µl of embryos):

1. Culture embryos in 0.5 ppm methylene blue solution at 28°C and collect them (100 – 200) just prior to hatching (7 days after fertilization, green hatching enzyme gland is clearly visible). Transfer them into a 2 ml eppendorf tube and wash them 4-5x in distilled water to remove methylene blue.
2. Remove all of the water
3. Shock freeze in liquid nitrogen
4. Thaw at 37°C, pass through 3 sucessive freeze /thaw cycles
5. Homogenize with pestle in eppendorf tube
6. Spin down 5 min full speed
7. Transfer supernatant to fresh tube on ice
8. Homogenize remainder again, spin for 45 sec full speed, combine supernatants
9. Homogenize pellet again with 500 µl PBS, spin 45 sec full speed and ccombine supernatants
10. Spin supernatant again 10 min at full speed and transfer the liquid phase to a new tube

11. The hatching enzyme can be used directly or be stored at -80°C after shock freezing in liquid nitrogen

### Removal of chorion

1. Wash the embryos in 2-3x in distilled water and transfer them to a 24-well tissue culture plate
2. ncubate embryos 40-60 minutes in 20 mg/ml Proteinase K in distilled water at RT in a limited volume on a rocking platform. (NB: the Proteinase K solution can be frozen and reused once.)
3. Wash embryos 3x in distilled water and transfer them to a 6 cm Petri dish. Remove all of the water.
4. Cover the embryos with a drop of hatching enzyme. Alternatively, use a 24- or 48-well tissue culture plate. In either case, take care that the embryos are arranged in a monolayer. If eggs are piled on top of each other, eggs at the bottom are crushed when the chorion dissolves.
5. Place a filter paper soaked in water into the lid of the Petri dish or tissue culture plate to prevent evaporation of the hatching enzyme.
6. Incubate at 28°C.
7. Check periodically under the stereomicroscope. Depending on the batch of hatching enzyme, hatching takes between 1-3 hours.
8. When the enzyme begins to work, a number of lunar crater-like holes open in the inner layer. The inner layer soon disappears, leaving only the soft outer chorion layer which can be easily removed with sharp forceps.
9. After this treatment, use sterile tools and reagents.
10. Add BSS/PEG/PS gently to float embryos.
11. Draw up embryos slowly using a wide-mouthed pipet (eg. Cell saver tips) and transfer gently to BSS/PEG/PS in a **non-adherent plastic suspension culture dish**. Alternatively, use a usual **Petri dish covered with 1 % agarose in BSS**, about 1 mm thick. Use this type of dish after this step to prevent dechorionated embryos from adhering to dishes.
12. Remove the outer layer of the chorion with sharp forceps sterilized in 70% alcohol.
13. Transfer dechorionated eggs to fresh BSS/PEG/PS with a wide-mouthed pipet.. The embryos will fall out of the pipet by gravity forces. Don't make dechorionated embryos come into contact with air. When they are drawn up with air bubbles, they collapse instantly.

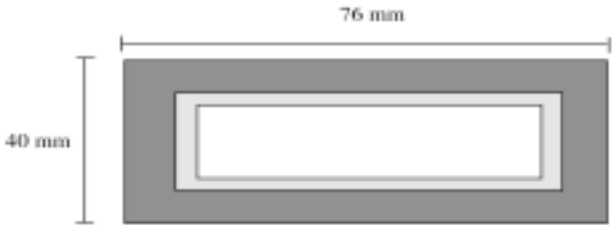
## Agar mounting

During gastrulation and body axis formation, medaka fish embryos show waves of contractile movements across the periderm, a tissue layer that covers both the developing embryo and its associated yolk cell. These contractions are generated by a layer of stellate-shaped cells closely apposed to the inner surface of the periderm (Cope et al 1990). In order to immobilize the embryos for microscopic observation, these movements can be inhibited by adding 3.5 mM 1-heptanol to the medium (Cope et al., 1990, Meda et al. , 1986). The effect of heptanol is reversible, thus all media including the embedding agarose must contain heptanol.

1. Transfer embryos to BSS/PEG/PS containing 3.5 mM Heptanol.
2. Incubate for 40 – 60 min until most of the contractile movements ceased.
3. In the meantime prepare a 0.2 - 0.5 % solution of low-melting point agarose in BSS and keep it at 37°C. Add 3.5 mM heptanol when the agarose cooled down to 37°C.
4. Prepare 1 % agarose/BSS coated slides as shown below.

5. Fill 0.5 % agarose into the depression of the slide and quickly transfer the embryo to the agarose.
6. Orient the embryo and let the agarose harden.
7. Cover the slide with BSS/PEG/PS, 3.5 mM heptanol and seal with a coverslip (24 x 60 mm).

## Viewing chambers




Plexiglass slide-form with a milling of 27 x 62 mm and a hole of 16 x 45 mm that will hold the coverglass of 24x60 mm


Plexiglass mold to make a depression into the agarose (24 x 60 mm)

**Viewing chambers:**
Seal a 24 x 60 mm coverslip to the slideholder with tape, fill the hole with 1 % agarose in BSS and insert the mold. Let the agarose harden.

## Material needed

## Buffers and solutions

### Balanced Salt Solution (BSS) <Heading 4>

Solution A (500 ml):

| | |
|---|---|
| NaCl | 65 g |
| KCl | 4 g |
| $MgSO_4 \times 7\ H_2O$ | 2 g |
| $CaCl_2 \times 2\ H_2O$ | 2 g |
| Phenol Red | 5 mg |

Autoclave and store at 4°C for up to 1 month; Phenol red can be omitted from the solution.

Solution B:
5 % $NaHCO_3$ in $dH_2O$, sterile filtered

Preparation of BSS:
Dilute 25 ml Solution A in 475 ml $dH_2$, add 1 ml Solution B to adjust the pH and sterile filter
→use this solution for preparing agarose in BSS

To culture embryos for extended periods, add 1 % PEG 20000 (or 8000) and Penicillin-Streptomycin (GibcoBRL, # 15140-122, 100 x stock) and sterile filter.

Store BSS at 4°C for up to 1 month.

### Heptanol (SIGMA, H-6129) stock solution (1000 x)

3.5 M 1-heptanol in DMSO. The endconcentration of DMSO in the medium will thus be 0.1 %.

### References


Yasumasu, S. et al. (1994). CDNAs and the genes of HCE and LCE, two constituents of the Medaka hatching enzyme. Develop. Grwoth & Differ. 36, 241-250

Cope, J. et al. (1990). The stellate layer and rhythmic contractions of the Oryzias latipes embryo. J. Exp. Zool. 254: 270-275

Meda, P. et al. (1986). Blockage of cell-to-cell communication within pancreatic acini is associated with increased basal release of amylase. J. Cell Biol. 103:475-483


# 4. MICROINJECTION AND OTHER TRANSFECTION METHODS

---

## 4.1 Experiment: Microinjection of DNA encoding GFP and sonic hedgehog transcripts under the control of a heat-shock inducible promoter, followed by simultaneous detection of GFP protein and sonic hedgehog RNA

(contributed by Carl Neumann)

Injection of DNA into fertilized zebrafish eggs leads to mosaic inheritance of the injected DNA, and can thus be used to drive the expression of specific transgenes in scattered cells or small groups of cells. To be useful, this experiment requires that we can identify the cells which are expressing the injected transgene. In this practical, we will test one possible approach to achieve this. We will inject a plasmid carrying two transgenes, GFP and sonic hedgehog, each under the control of a heat-shock inducible promoter. After activating expression by exposing the injected embryos to heat shock, we will use fluorescent immunohistochemistry to detect the GFP protein, and in situ hybridization with a sonic hedgehog probe, followed by detection using the fluorescent substrate Fast Red. This will allow simultaneous detection of the two gene products using confocal microscopy, and will reveal whether the two genes are co-misexpressed.

### Overview:

- Inject plasmid DNA into freshly laid fertilized eggs

- Heat-shock injected embryos to activate the transgenes
- Select GFP-expressing embryos and fix
- Perform whole-mount in situ hybridization with a fluorescent substrate to detect *sonic hedgehog* transcripts and fluorescent whole-mount immunohistochemistry to detect GFP protein
- Analyze embryos on Leica confocal microscope

## Microinjection of plasmid DNA

1. Break off the tip of a needle to be used for injection using forceps under a dissecting microscope
2. Load the needle with circular plasmid DNA (30ng/□l)
3. Attach loaded needle to a microinjector
4. Place an injection mold with medium undrneath the needle
5. Dip needle into the medium, and observe it with the stereo microscope. If necessary, adjust setting of the the microinjector such that no liquied is sucked up into the needle, and none leaks out.
6. Place freshly laid zebrafish eggs in the injection mold, and move the eggs into the trenches of the injection mold using forceps
7. Now bring a few eggs into focus, and adjust the injection needle so that it points at one of these eggs, ready to be stabbed into it
8. Orient the egg such that the large cell on top of the yolk is pointing towards the needle, and push this egg into the agarose of the injection mold to stabilize it
9. Readjust needle using the micromanipulator, and stab it through the chorion into the large oocyte cell
10. Eject a small volume of DNA solution into the cell, and withdraw needle
11. After injecting a number of oocytes, move them from the injection mold to a petridish containing hatching medium with a plastic pipette. Incubate at 28 degrees overnight

## Heatshocking of the embryos

1. Place the whole petridish containing injected embryos in a 37 degree incubator for 2 hours
2. Allow recovery for at least 2 hours
3. Examine embryos under a stereo microscope fitted with a fluorescent lamp and a GFP filter
4. Identify embryos with GFP-expressing cells, and place these embryos in a 2 ml eppendorf tube for fixation

## Fixation/permeabilization

1. Fix embryos with 4% PFA in PBS 1 hour RT or o/n at 4 degrees
2. Rinse with PBS
3. Transfer to petridish with PBS. Dechorionate with forceps
4. Transfer to 0.7 ml eppendorf
5. Replace PBS with methanol and leave for 5 min
6. Replace with new methanol and place at –20 degrees for at least 0.5 hours

## Postpermeabilization/proteinase K treatment

1. Replace half of the methanol with PBS and leave for 5 min at RT
2. Repeat step 1

3. Replace all of the liquid with PBST and leave for 5 min
4. Digest with 5□g/ml proteinase K (use 20mg/ml glycerol stock and add 0.25 □l to 1 ml PBST). Digest 10 min
5. Rinse 3 times with PBST

## Prehybridization/Hybridization

1. Replace PBST with 200 □l Hyb-. Incubate at 67 degrees for 5 min
2. Replace Hyb- with 200 □l Hyb+. Prehybridize for 1 hour at 67 degrees
3. Take off 120 □l of Hyb+ withour letting the embryos touch air
4. Add 1 – 2 □l of probe to the sample (see protocol by M. Carl on how to make in situ probes)
5. Incubate o/n at 67 degrees

## Probe removal/washes

1. Remove probe solution and wash 2 x 30 min with 50% formamide/2X SSCT at 67 degrees
2. Wash 1 x 15 min with 2X SSCT at 67 degrees
3. Wash 2 x 30 min with 0.2X SSCT at 67 degrees

## Detection (Fast Red)

1. Rinse once with 1X Malate butter
2. Block at least 1 hour at RT with 1X Malate buffer plus 2% Roche blocking reagent
3. Add alkaline phosphatase-coupled mouse anti-DIG antibody at 4000-fold dilution. Add 0.5 □l to 2 ml 1X Matate buffer plus 2% block. Also add primary rabbit anti GFP antibody at a 500-fold dilution
4. Incubate 4 hours RT or o/n at 4 degrees
5. Rinse 2X with 1X Malate buffer
6. Wash 2X 45 min with 1X Malate buffer
7. Rinse 2X with staining buffer pH 8
8. Wash 1X with staining buffer pH 8 for 15 min
9. Dissolve 1/2 Fast Red tablet per 2 ml staining buffer pH8 using pipette tip
10. Vortex briefly
11. Spin the solution at highest setting for 5 min
12. Transfer the solution to a new tube, leaving behind the undissolved particles
13. Incubate the embryos in this solution for 15 min to several hours at 37 degrees
14. Inspect embryos periodically under a Stereo Microscope. When the reaction is complete, rinse several times with PBST
15. Rinse 1X with Malate buffer
16. Block 0.5 hours with 1X Malate buffer plus 2% blocking reagent
17. Add secondary goat anti rabbit antibody conjugated with Alexa488 at a 50-fold dilution (add 10 □l to 500□l)
18. Incubate 2 hours RT to o/n at 4 degrees
19. Rinse 3X with PBST
20. Wash 2X 45 min with PBST
21. Fix 20 min with 4% PFA in PBS
22. Equilibrate embryos in 80% glycerol with PBST
23. Mount on a microscope slide and analyze with a confocal microscope

## List of material needed

- Stereo Dissecting Microscope (Zeiss)
- Stereo Dissecting Microscope with Hg-lamp and filter for detecting GFP fluorescence (Leica)
- Confocal Microscope (Leica)
- Eppendorf Femtojet Microinjector
- Micromanipulator for injection needle (Eppendorf, Leica)
- Rabbit anti-GFP antibody (Torrey Pines Biolabs)
- Goat anti-rabbit antibody coupled to Alexa488 (Molecular Probes)
- Mouse anti-DIG antibody coupled to alkaline phosphatase (Roche)
- Fast Red tablets (Roche)

## Buffers and solutions

### PBST

PBS plus 0.1% Tween-20

### SSCT

SSC plus
Tween-20 0.1%

### Hyb-

Formamide 50%
SSC 5x
Tween-20 0.1%
(Store at -20$^{o}$C)

### Hyb+

Hyb-
Heparin 50 mg/ml
torula (yeast) RNA 5 mg/ml
(Store at -20$^{o}$C)

### Staining buffer

| | | |
|---|---|---|
| Tris pH 8-8.2 | 100mM | 50 ml 1M stock |
| MgCl2 | 50 mM | 25 ml 1M stock |
| NaCl | 100mM | 16.7 ml 3M stock |
| Tween-20 | 0.1% (add just before use) | |
| | + 408.3 ml $H_20$ | |

## 4.2 (Alternate protocol) Injection of RNA and DNA into zebrafish embryos

(E. Raz, Göttingen)

### Experiment description:

GFP as mRNA and in a DNA expression construct will be injected into early zebrafish embryos. Expression of GFP in the injected embryos will be followed and will be compared between the DNA and RNA injected fish. Injection of b catenin and of wnt8 mRNA will allow for biological effects of injected RNA to be observed. The development of fish injected with the different RNAs will be observed in live embryos and in fixed embryos stained with gsc and ntl probes.

### Time table:

day 1 afternoon - prepare injection ramps and micropipets. Set up crosses
day 2 morning - harvest eggs and inject.
follow the development of the injected embryos and look for GFP expression.

### Supplies needed:

- Microinjector (Eppendorf)
- Micropipets
- Micropipet puller
- Microloader pipet tips (Eppendorf)
- Micromanipulator (Leica, Eppendorf)
- Dissecting scope (Zeiss)
- Glass Petri dish

### Reagents needed:

1. 0.3X Danieau's

A 30 X Danieau's solution can be made (dilute it 1:100 before use)-

1.74 M NaCl
21 mM KCl
12 mM $MgSO_4$

18 mM $Ca(NO_3)_2$

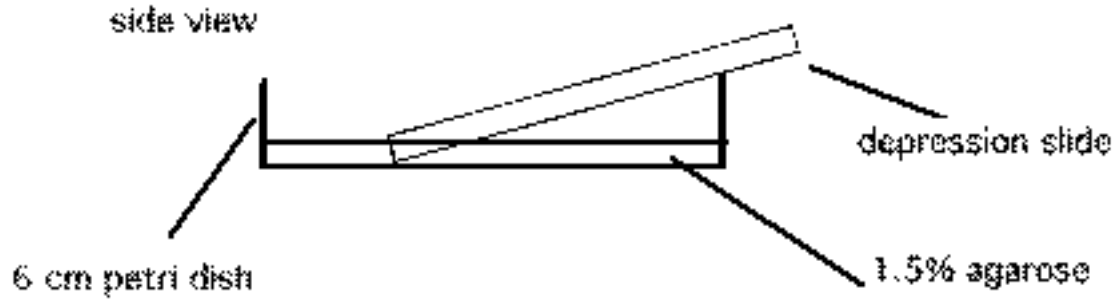
150 mM HEPES

pH 7.6

2. 0.3 X Danieau's + 1% penicillin streptomycin
3. DNA 25-50 ng/ml or RNA 5-50 ng/ml
4. 4 mg/ml pronase E in 0.3X Danieau's
5. Agarose

## Pre injection preparations:

1. Boil 1.5% agarose in 0.3X Danieau's.
   Use the agarose to coat the plastic dishes that will be used to keep the embryos after injection. After the agar cools down, cover with 0.3 X Danieau's
2. Prepare injection ramps. Place a thick depression slide in a 6 cm petri dish and add agarose. After the ramp solidifies cover with 0.3 X Danieau's.




3. pull micropipets and break the tip using a razor blade if the tip is too thin

## Dechorionization:

Place the embryos in a 6 cm agarose coated petri dish, remove the liquid and add 10 ml of 4mg/ml pronase. Observe under dissecting scope. When 3-4 embryos come out of the chorion (in 2-3 minutes) start washing in a 10cm glass petri dish. Wash by changing the liquid over the embryos 8-10 times. Most of the embryos should come out of the chorion during the washes.

Transfer the dechorionated embryos onto the ramp. About 30 embryos will fit as one row on one ramp.

## Injection:

Using a microloading pipet tip put 1-3 ml of the RNA or DNA solution in the pulled micropipet. Adjust the back pressure so you get a very slow outflow of solution out of the pipet and adjust the time and injection pressure so it delivers the right amount to be injected. Inject embryos and then using pastuer pipettes transfer them to agarose coated plates containing 0.3 X Danieau's + 1% penicillin streptomycin.

## 4.3 Injection of DNA/RNA/morpholinos/tracer dyes into early medaka embryos

(M. Carl and J. Wittbrodt)

Medaka egg clusters are collected from females 30 min after light turns on and are transferred to a petri dish containing hatching solution. Eggs are separated by gently rolling them in the dish or on a Whatman paper. Remaining traces of attachment filaments are removed manually with forceps under a dissecting microscope. Embryos can be cooled down to 4°C for several hours to slow down development if a big number of embryos needs to be injected. Isolated embryos are transferred to agar coated Petri dish (1.5% Agarose in water) filled with 1x Yamamoto Ringer with grooves 1 mm wide, 0.9 mm deep to fix the embryos.

In Medaka, it is not necessary to remove the chorion prior to microinjection as it can be easily penetrated with the injection needle. However, embryos tend to move inside the chorion, so that each embryo has to be oriented properly just prior to the injection.

### Needles:

Filament containing borosilicate glass capillaries (Clarc Electromedical Instruments, GC100F-10) are routinely used. Needles are pulled on a Sutter needle puller with a closed sharp, but rigid tip. Prior to filling the needles the back ends are briefly fire polished. Needles are filled from the back with Eppendorf microloaders. The needle tip is opened under water by gently touching it with a pair of sharp forceps. For the Eppendorf microinjetor the starting settings are 80-100 hPa for holding pressure and 500-700 hPa for injection pressure. The optimal injection time (to inject 15-20% of the cell volume) is established empirically.

### DNA injection:

App. 500pl (= 1/6 of the cell volume) of plasmid-DNA at a concentration of 10-50µg/ml (25pg DNA in total injected per cell) are injected into the cytoplasm of early embryos (usually at the 2-cell stage, because this is easiest to inject). The DNA injection solution is in 1x Ringer (that can contain 0.2% phenol red).

### RNA/morpholino/tracer injection:

Capped mRNA is synthesized in-vitro using an Ambion "mMessage mMachine" Kit. The RNA is injected in 1xRinger´s solution at concentrations of 50µg/ml up to 1mg/ml (i.e. from 25pg to 500 pg RNA per cell).

Tracer-dyes (like FITC-dextran; Rhodamine-Dextran) are injected at a concentration of 1.5% in 1x Ringer´s solution.

Injected embryos are transferred to hatching solution and kept at 28°C till hatching.

## Buffers and solutions

### 10x (Yamamoto's isotonic BSS; Ringer´s solution):

| | |
|---|---|
| NaCl | 7.5 g |
| KCl | 0.2 g |
| $CaCl_2$ | 0.2 g |
| $NaHCO_3$ | 0.02 g |

to a final volume of 100 ml with distilled water; adjust pH to 7.3

### Hatching solution:

| | |
|---|---|
| NaCl | 0.1% (w/v) |
| KCl | 0.003% (w/v) |
| $CaCl_2$ x 2 $H_2O$ | 0.004% (w/v) |
| $MgSO_4$ x 7$H_2O$ | 0.016 % (w/v) |
| methylene blue | 0.0001% (w/v) |

## References:


Winkler, C., Wittbrodt, J., Lammers, R., Ullrich, A. and Schartl, M. (1994). Ligand-dependent tumor induction in medakafish embryos by a Xmrk receptor tyrosine kinase transgene. Oncogene *9*, 517-1525.

Wittbrodt, J. and Rosa, F. M. (1994). Disruption of mesoderm and axis formation in fish by ectopic expression of activin variants: The role of maternal activin. Genes & Development *8*, 1448-1462.

Oliver, G., Loosli, F., Köster, R., Wittbrodt, J. and Gruss, P. (1996). Ectopic lens induction in fish in response to the murine homeobox gene *Six3*. Mech Dev *60*, 233-239.

Köster, R., Stick, R., Loosli, F. and Wittbrodt, J. (1997). Medaka spalt acts as a target gene of hedgehog signaling. Development *124*, 3147-3156.


---

# 4.4 Highly efficient transgenesis in fish mediated by *I-SceI* meganuclease

(contributed by Clemens Grabher, Felix Loosli, Jochen Wittbrodt)

The widespread use of fish as model systems is still limited by the mosaic distribution of cells transiently expressing transgenes leading to a low frequency of transgenic fish. Here we present a strategy that overcomes this problem. Transgenes of interest are flanked by two *I-SceI* meganuclease recognition sites, and co-injected together with the *I-SceI* meganuclease enzyme into medaka embryos (*Oryzias latipes*) at the one-cell stage. By this approach, the promoter dependent expression in F0 is

strongly enhanced and the transgenesis frequency is raised to more than 30%. In addition, the average germline transmission rate (the number of transgenic F1 offspring of an identified founder fish) approaches the maximum of 50% for single insertions indicating an integration event already at the one cell stage.

Prepare injection plates (1.5% agarose/water)

1. Collect eggs immediately after spawning (at the onset of light) and placed in pre-chilled Yamamoto's embryo rearing medium or Hanks (not pre-chilled !!) in case of zebrafish. Roll medaka clutches to separate them into single embryos
2. Arrange single embryos appropriately in injection plates.
3. Medaka embryos may be put to 4°C to arrest development. Let develop to one-cell stage.
4. Backfill injection needles with injection solution using GELoader tips and mount to injector.
5. Inject DNA through the chorion into the cytoplasm (not yolk!) of a one-cell stage embryo. Injection volume should not exceed 10% of the cell volume.
6. Monitor GFP fluorescence according to the promoter used between 8 hrs and 3 days after injection. Change Yamamoto buffer daily.
7. To identify stable transgenic fish, raise GFP expressing F0 fish to sexual maturity and mate to wild type fish.

## List of material needed

- Medaka orange-red or Cab strain
- Zebrafish AB strain
- 10 cm bacterial dishes
- Injection plates
- Pressure Injector (Femtojet, Eppendorf, Germany)
- Borosilicate glass capillaries (GC100F, Clark Electromedical Instruments, UK)
- Wide-mouthed pipettes
- Forceps (INOX 5)
- Microloader tips (Eppendorf, Germany)
- Casting molds

## Buffers and solutions

### Injection solution (15µl)

| | |
|---|---|
| DNA (Qiagen Maxipreps) | 10ng/µl |
| Meganuclease buffer (NEB or Roche) | 0.5x |
| I-SceI meganuclease | 0.2 units/µl |
| Distilled water | ad 15µl |

### Yamamoto buffer (1l)

| | |
|---|---|
| NaCl | 7.5g |
| KCl | 0.2g |
| $CaCl_2$ | 0.2g |
| pH | 7.3 |
| Distilled water | ad 1l |

autoclave

1.5% agarose in water

| | |
|---|---|
| Agaraose (ultraPure, GibcoBRL) | 6g |
| Distilled water | ad 400ml |

## References

Yamamoto, T. (1975) Medaka (killifish): biology and strains. Heigaku, Tokyo

Violette Thermes*, Clemens Grabher[1]*, Filomena Ristoratore#*, Franck Bourrat, André Choulika[2], Jochen Wittbrodt[1], Jean-Stéphane Joly (2002) *I-SceI* meganuclease mediates highly efficient transgenesis in fish. Mechanisms of Development, in press

---

# 4.5 Knock-down of maternal factors by nuclear injection of morpholinos into premature medaka oocytes

(contributed by C.Winkler)

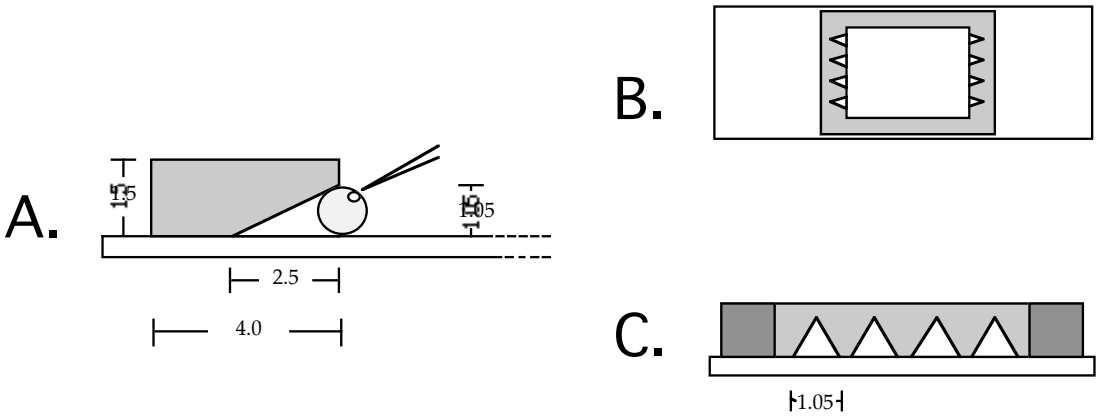
Medaka offers the unique possibility to maturate oocytes in-vitro using standard tissue-culture media. This allows the isolation of premature oocytes at a specific stage during oogenesis when the germinal vesicle is apparent. During a window of 2 to 3 hours the large and clearly visible germinal vesicle (GV) can be injected with a solution containing plasmid DNA, RNA or morpholinos. After injection the oocytes are cultured in a basic medium, where GV breakdown and the late stages of oogenesis occur during maturation. In medaka, contrary to other fish species, these steps are apparently independent from the direct contact of oocytes to the supply of hormones in the ovary. After 8 hrs in culture, the isolated oocytes spontaneously ovulate and are then ready for in-vitro fertilization. In the basic medium used they stay fertilizable for several hours (I checked fertilization 12 hours after the scheduled time and did not find significant reduction of survival rates). The fact that plasmid DNA is injected directly into the oocyte nucleus, where it stays for at least 9 hours before the first cell division, apparently increases the chance for integration into the host genome and for contribution to the germ line.

The method of nuclear oocyte injection and the conditions for in-vitro maturation to generate stable transgenic medakafish has been originally described by Ozato in 1986. Thereafter, a number of transgenic lines have been established where various transgenes were successfully transmitted to progenies and properly expressed.

Two experiments concerning nuclear transfer of morpholinos and RNA will be performed during this EMBO course. First, a morpholino oligo directed against medaka β-catenin will be injected at different concentrations into immature oocytes. These oligos interfere with β-catenin translation and result in a reduction of maternal β-catenin protein pools. The consequences of this on dorsoventral patterning of the embryonic axis will be analyzed morphologically after in vitro fertilization. A rescue of this "morphant" phenotype will by attempted by coinjection of RNA encoding Xenopus β-catenin into some control oocytes. In a second set of experiments, RNA encoding a GFP fusion with DMRT-1 will be injected as described above. The encoded fusion protein localizes to chromatin and will allow to follow nuclear morphology during different phases of oocyte maturation, fertilization and cell divisions.

## Isolation of premature oocytes

1. Adult fish are kept under standard conditions and a photoperiod of 14 hrs light and 10 hrs dark
2. Spawning females are killed by decapitation 9 hrs before the anticipated onset of light phase
3. Ovaries are carefully removed without distroying the surrounding epithelia and transferred to 60 mm dishes containing oocyte medium.
4. Premature oocytes with clearly visible nuclei are carefully separated from immature oocytes and ovary tissue. Completely remove all tissue that is attached to oocytes. Be careful as premature oocytes are extremely fragile. Transfer oocytes to holding tray filled with oocyte medium. Carefully position oocytes into the grooves of the holder and orientate the oocytes with the nucleus facing upwards.




Oocyte injection tray made of transparent plastic. A specially designed plastic rim containing notches as indicated is glued onto a plastic or glass slide using "instant glue". A. Lateral view. B. Top view. C. Front view. All sizes in mm.

## Nuclear injection into medaka oocytes

1. Inject oocytes between 9 and 6 hrs before the onset of light phase. Although the nucleus is visible earlier than that, oocytes isolated earlier will not mature successfully in-vitro. At 6 hrs before light-on germinal vesicle break down (GVBD) occurs and the nucleus becomes invisible.
2. Inject morpholinos at concentrations between 1 and 10µg/µl and RNA (at 0.5µg/µl) into the vicinity of a clear vesicle inside the nucleus. As soon as you see slight movement of vesicles stop injecting. Oocytes are very sensitive to large volumes of injected solutions. Try to inject a minimum amount of solution to decrease lethality. Make sure not to inject into the yolk or cytoplasm by controlling movement of vesicles inside the nucleus.
3. Using a pipette with wide opening carefully transfer injected oocytes to fresh oocyte medium and let them mature at 28°C.

4. At the onset of light phase ovulation occurs spontaneously (if not, the follicular layer needs to be removed manually with fine forceps).
5. Isolate testis from adult males that were separated from females the day before injection.
6. In-vitro fertilize oocytes by mincing testis with forceps and mixing sperm suspension with oocytes in as little volume of oocyte medium as possible; use 1 testis per 1 ovary used; check mobility of sperm.
7. Incubate sperm with oocytes for 30 minutes at 28°C; check cortical reaction
8. Thoroughly wash fertilized eggs 3 times with Embryo Rearing Medium. Remove follicular tissues and testis.
9. Incubate embryos in embryo rearing medium at 28°C till hatching. The medium should be changed daily if residual follicular tissue is present and bacteria start to grow (though, medaka eggs tolerate some contamination!). Check phenotypes of morpholino injected embryos during gastrulation and neurulation stages. Analyze GFP expression in GFP::DMRT-1 injected oocytes during maturation, pre- and post-fertilization stages.

## Buffers and solutions

Earle's Medium 199; Gibco BRL (US# 31100-035), powdered, for 1 liter .

Gibco Pen-Strep solution (1 small bottle)

2 bottle-top filters (0.2$\mu$m; 500ml)

## References


Hyodo-Taguchi, Y., Winkler, C., Kurihara, Y., Schartl, A. & Schartl, M. (1997). Phenotypic rescue of the albino mutation in the medakafish (*Oryzias latipes*) by a mouse tyrosinase transgene. Mech. Dev. 68, 27-35.

Ozato, K., Wakamatsu, Y. & Inoue, K. (1992). Medaka as model of transgenic fish. Mol. Mar. Biol. Biotech. 1, 346-354.

Ozato, K., Inoue, K., and Wakamatsu, Y. (1989). Transgenic fish: biological and technical problems. Zool. Sci. 6, 445-457.

Ozato, K., Kondoh, H., Inohara, H., Iwamatsu, T., Wakamatsu, Y., and Okada, T.S. (1986). Production of transgenic fish: introduction and expression of chicken delta-crystallin gene in medaka embryos. Cell Differ. 19, 237-244.

Takagi, S., Sasado, T., Tamiya, G., Ozato, K., Wakamatsu, Y., Takeshita, A. & Kimura, M. (1994). An efficient expression vector for transgenic medaka construction. Mol. Mar. Biol. Biotech. 3, 192-199.

Toyohara, H., Nakata, T., Touhata, K., Hashimoto, H., Kinoshita, M., Sakaguchi, M., Nishikimi, M., Yagi, K., Wakamatsu, Y. & Ozato, K. (1996). Transgenic expression of L-gulono-gamma-lactone oxidase in medaka (*Oryzias latipes*), a teleost fish that lacks this enzyme necessary for L-ascorbic acid biosynthesis. Biochem. Biophys. Res. Comm. 223, 650-653.


---

# 4.6 Monitoring specification of cell movements and fates in zebrafish gastrula

(contributed by Lila Solnica-Krezel)

During gastrulation cell cells engage in gastrulation movements shaping the vertebrate body plan while their fates become specified. The relationships between determination of cell fate and movement behaviors are only poorly understood. It is known that in the fish gastrula, prospective mesendodermal cells at the blastoderm margin undergo involution/ingression to underlie ectodermal layer. Furthermore, all germ layers engage in convergence and extension movements that elongate the nascent embryonic axis from head to tail and narrow it mediolaterally.

Tracking dorsal translocation (convergence) or anteroposterior elongation (extension) of cell groups labeled with either lipophilic or photoactivatable dyes revealed the presence of three C&E movement domains in the zebrafish gastrula (Myers et al., 2002). First, the most ventrally located mesodermal cells do not converge but rather spread over the yolk, later migrating into the tailbud region without extension. Such cell behavior typifies cells residing within a 20-30˚ arc of the ventral margin, termed the *no convergence no extension zone* (*NCEZ*). Second, in the lateral domain, convergence and extension movements are initially slow, increasing as cell groups move dorsally. Third, in the dorsal domain labeled cell populations exhibit strong extension with little convergence. These findings support a new model of C&E at the cell population level whereby ventrally, convergence and extension are absent. Laterally, they occur at increasing rates and dorsally, strong extension movements are accompanied by limited convergence.

Marginal cell populations positioned at the beginning of gastrulation 90˚ from the embryonic shield, at the onset of somitogenesis form anterposterior arrays located close to the dorsal midline. These cell arrays are found just at the lateral boundary of the first forming somites and within the presomitic mesoderm.

What are movements and fates of latero-ventral cell populations located between the boundary of the ventral NCEZ and the 90˚ position? We will address this question by first injecting wild-type zebrafish zygotes with caged fluorescein dye and then photoactivating the dye in small cell populations located in the ventrolateral margin at early gastrulation (shield stage). We will determine the exact position of the labeled cell population with respect to the embryonic shield upon photoactivation and allow embryos to complete gastrulation. At the onset of somitogenesis embryos will be fixed and the labeled cell populations will be visualized using anti-Fluorescein Antibodies. We will assess fates of the labeled cells by simultaneously monitoring expression of intermediate and lateral plate mesoderm markers (*pax2.1*, *gata2*) by whole mount in situ hybridization.

## Protocol:

## Injection and Uncaging of Fluorescein for Fate Mapping and Extension Measurement

1. Dextran DMNB Caged fluorescein 10,000 MW (Molecular Probes D-3310) was dissolved in 120 mM KCl, 20 mM Hepes pH 7.5 to a final concentration of 1% [Kozlowski, 1997 #2733]. Before use, the dye was centrifuged 3-5' in a microfuge.
2. A small quantity (0.5 to 1 nl) was injected through the chorion into the yolk of an 1-8 cell stage embryo using a pneumatic picopump (WPI) as described in Marlow et al. (1998).
3. Following injection, embryos were sorted by stage and kept in the dark at $28.5^0$.
4. Embryos were manually dechorinated in sterile filtered embryo medium and mounted at shield stage on bridge slides in 2.5% methyl cellulose in embryo medium with 10mM HEPES [Cooper, 1998 #2532].
5. We uncage a small spot of dye at shield stage on the margin [Kozlowski, 1997 #2733] on an Olympus AX70 microscope, using DAPI filter epifluorescence, through a 40X objective and a stopped down epifluorescence iris diaphragm. The location of the spot relative to dorsal was determined using an eyepiece protractor reticle (Klarmann Rulings).

## Labeling uncaged dye after In Situ Hybridization

1. Prepare an in situ hybridization according to the Thisse protocol with a probe labeled with digoxygenin and a probe labeled with FITC. Prepare two sets of blocking embryos to pre-clear

both anti dig and anti-FITC antibodies at dilutions of 1:1000 in PBST, 2% sheep serum, 2 mg/ml bovine serum albumen. Use antibodies at 1:5000 dilution.

2. After coloring the in situ hybridization embryos, strip off the dig antibody with 0.1 M glycine pH 2.2, 0.1% tween-20, 3x for 5 minutes.
3. Wash 3x 5 minutes with PBST.
4. Bind 1:5000 dilution anti-FITC Ab to experimental embryos for 2 h at RT.
5. Wash Ab 6 x 15 minutes in PBST.
6. Wash 2 x 15 minutes in 0.1 M TRIS pH 8.2, 0.1% Tween-20.
7. Prepare Fast Red solution. Dissolve the fairly insoluble tablet in 0.1 M TRIS pH 8.2, 0.1% Tween-20 with vortexing. Remove particles from the solution either by using a syringe filter or by spinning the particles down using a microfuge.
8. Stain embryos in Fast Red solution at RT. The color will develop in about 5-10 minutes for uncaged FITC labeled cells.

## Material needed

Dextran DMNB caged fluorescein 10,000 MW (Molecular Probes D-3310)

Antifluorescein alkaline phosphatase, Fab fragments (Boehringer Mannheim Biochemicals cat#852-631-24-06)

Fast red tablets (Boehringer Mannheim Biochemicals cat#1-496-549)This is only 20 tablets.

We used a protractor reticle (Klarrman Rulings) we purchased from Zeiss. Klarrman can be found on the Web, however, asKlarrman is a US company, it would probably be easier to obtain a reticle locally

## Buffers and solutions

120 mM KCl, 20 mM Hepes pH 7.5

0.1M glycine pH 2.2, 0.1% tween 20

100% Danieau's buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM Hepes, pH 7.6)

2.5% methyl cellulose in embryo medium with 10mM HEPES . This hydrates slowly so start at least 2 days before

## References:


Myers et al., 2002.

Kozlowski, D. J., Murakami, T., Ho, R. K., and Weinberg, E. S. (1997). Regional cell movement and tissue patterning in the zebrafish embryo revealed by fate mapping with caged fluorescein. Biochem. Cell Biol. 75, 551–562.

Marlow et al. (1998).

Cooper, 1998

Thisse, 1998

# 4.7 (Alternate Protocol) Fate mapping by local activation of photoactivable (caged) fluorescein

(contributed by Laure Bally-Cuif)

This technique allows the irreversible labeling of a group of cells at a desired stage and location, permitting to follow cell fate over development. It uses the local activation of a photoactivatable dye that is injected into the embryo at an early stage and becomes fluorescent when activated by specific wavelengths of UV light. The UV beam is produced by the epifluorescence illumination pathway of a microscope, narrowed by a pinhole, or is produced by a laser. In the first case, a 0.15 mm diameter pinhole inserted at the focal plane into the path of the UV beam will allow to activate 10-50 cells at the gastrula stage with a 40x objective. When using a laser, single cells can be activated. Activated cells can be followed on live specimen by fluorescence microsocopy. In addition, immunocytochemical detection of cells containing uncaged fluorescein using an anti-fluorescein antibody can provide a stable record of cell distribution.

## Procedure for uncaging

1. Prepare the injection box: boil 1.2% agarose in embryo medium, pour in Petri dishes and overlay with the injection molds; let set then remove the mold and cover with embryo medium;
2. Prepare the uncaging boxes: boil 1.2% agarose, pour in a glass dish and overlay with the transplantation mold; let set then remove the mold and cover with embryo medium + penicillin/streptomycin;
3. Align embryos (in their chorion) in the injection rows, yolk cell on the pipette's side;
4. Protect the lights of the room, as well as that coming from the stereomicroscope halogen lamp, with UV-cut filters;
5. Inject the caged fluorescein (5-10 mg/ml in Ambion $H_2O$) at the 1-cell stage, approx. 10 pl per embryo (settings of the microinjector: Pi 100-300 hPa; manual function for the time setting);
6. Transfer injected embryos into a fresh Petri dish of embryo medium and let develop at 28.5°C in the dark until the desired stage;
7. Still using UV-cut filters, dechorionate the injected embryos under a stereomicroscope using fine forceps, and dispose them into the transplantation holes, side to be activated up; transfer to the microscope stage;
8. Protect the halogen illumination of the microscope with UV-cut filter, then, with a 40x (or 50x) water objective, focus on the cell group to be activated;
9. Insert the pinhole holder into the epifluorescence light pathway;
10. Switch off the halogen illumination, switch on the mercury lamp and, by looking through the FITC filter set, move the stage so that the cells to be irradiated (still in focus) are under the light beam;
11. Switch the filter set to the DAPI filter and irradiate for 2-4 sec.;
12. To visualize the irradiated cells, switch the filter set back to the FITC filter and remove the pinhole; to avoid background, do not observe for longer than a few seconds!
13. Remove the glass dish from the stage and let the embryos develop in their holes in the dark to the desired stage;
14. Fix overnight at 4°C in 4% PFA;
15. Rinse in PBT, then dehydrate in 50% PBT;50% MeOH → 20% PBT;80% MeOH → 100% MeOH (twice); store at –20°C.

## Procedure for anti-fluorescein immunocytochemistry

1. Rehydrate to PBT in a reverse MeOH series;
2. Incubate in $H_2O$ for 1 hr at room temperature;
3. Transfer to eppendorf tubes, rinse in acetone then incubate in acetone for 7 min. at –20°;
4. Rinse several times in $H_2O$ then transfer back to PTD;
5. Block 1 hr at RT in PTDBN;
6. Incubate overnight at 4°C in sheep anti-fluorescein-HRP antibody at a final dilution of 1/200 in PTDBN;
7. Rinse 5 times 10 min. at RT in PTD (optional: rinse then overnight at 4°C) with gentle rocking;
8. Preincubate in 100 mg/ml DAB in 0.1 M Tris-HCl pH 7.5
9. Add $H_2O_2$ to a final concentration of 0.03% and monitor the color reaction under a stereomicroscope
10. When staining of a desired intensity is obtained, rinse the embryos at least 5 times 10 min. in PTD at RT, then overnight in PTD at 4°C;
11. Transfer to 80% glycerol, mount and observe under the microscope.

## Material needed

- Anesthetic: Tricaine (3-amino benzoic acidethylester, Sigma A-5040), 25x stock is 4 mg/ml Tricaine in 20 mM Tris-HCl pH 7.5; dilute in embryo medium for use
- Injection mold (for injections rows and for transplantation holes) and agarose to make injection box
- DMNB-Caged fluorescein 10,000 MW (Molecular Probes D-3310) diluted to 20 mg/ml in Ambion $H_2O$ (stock solution, keep in the dark at –20°C)
- UV-cut filter (Encapsulite, ref. G10)
- Pipette puller and micropipettes
- Microinjector
- 0.1-0.15 mm stainless steel pinhole
- Microscope with epifluorescence (100W Hg-arc lamp and DAPI filter set)
- Embryo medium containing 1x penicillin/streptomycin (100x pen/strep stock Gibco ref. 15140-148)
- 4% paraformaldehyde (PFA) in PBS
- PBT (PBS; 0.1% Tween-20)
- PTD (PBS; 0.3% Triton x100; 1% DMSO)
- PTDBN (PTD; 2 mg/ml BSA; 1% Normal Goat Serum)
- Sheep anti-fluorescein-HRP antibody, Fab fragments (Roche ref. 1.426.346), preadsorbed at 1/50 on fixed control embryos in PTDBN
- 1mg/ml DAB stock (in $H_2O$)
-

## References

Kozlowski and Weinberg, In Methods in Molecular Biology, Vol. 135: Developmental Biology Protocols Vol.1 pp.349-355; R.S. Tuan and C.W. Lo eds, Humana Press Inc.(2000).

## 4.8 Retrograde labeling of reticulospinal neurons

(contributed by Laure Bally-Cuif)

This technique uses the retrograde propagation of lysinated rhodamine dextran to label, from a spinal lesion, all neurons projecting to the spinal cord. The reticulospinal complex offers an excellent assay for normal hindbrain / ventral midbrain development as many of the neurons of this complex are individually identifiable and are arranged in a segmental manner that reflects their identity.

### Procedure

1. Anesthetize 4- to 5-day old "larvae" with Tricaine-containing embryo medium
2. Pipet the larvae separately to the bottom of an empty Petri dish, one larva per drop of embryo medium; pipet out the medium drop and immobilize the larvae by adding on top a drop of liquid1% LMP agarose; let set
3. Cut off the tail at the level of the anus using a fine sharp tool previously plunged into the 5% lysinated rhodamine dextran solution (label the tool again for each fish)
4. Let the larvae stand for a few more minutes under the agarose drop
5. Remove the larvae from the agarose and let them recover for 1 hr in embryo medium
6. Fix overnight in 4% PFA at 4°C
7. Tranfer to PBS and dissect out the brains using fine forceps
8. Rinse further 2x 5 min in PBS then clear stepwise in glycerol:PBS (50%, 80%)
9. Mount in 80% glycerol between slide and coverslip, ventral side up, with the thickness of a single coverslip as a spacer
10. Observe under fluorescence standard or confocal microscopy

### Material needed

- Lysinated rhodamine dextran, fixable (Molecular Probes D-1817), 5% stock in $H_2O$
- 1% low melting point agarose in embryo medium, kept at 42°C
- sharp beveled needle or fine blade
- 4% paraformaldehyde (PFA) solution in PBS
- Anesthetic: Tricaine (3-amino benzoic acidethylester, Sigma A-5040), 25x stock is 4 mg/ml Tricaine in 20mM Tris-HCl pH 7.5; dilute in embryo medium for use

### References

Kimmel et al., *J. Comp. Neurol*. 205, 112-127 (1982)

Alexandre et al., *Development* 122, 735-746 (1996)

Moens et al., *Development* 122, 3981-3990 (1996)

## 4.9 Conditional missexpression by electroporation

(contributed by Laure Bally-Cuif)

This technique allows the ectopic expression of a gene of interest from a desired stage. It can be performed by incubation of entire embryos into the DNA solution at the time of electroporation; in that case essentially external tissues (ectoderm) are targeted. Alternatively, the DNA solution can be injected at any stage into the extracellular space, targeting internal tissues. When performed at late stages (i.e. after 24 hpf), the DNA can also be targeted to specific territories by local extracellular injection, e.g. to the anterior neural tube. Ubiquitous promoters such as *CMV* or *CSKA* are generally used to drive expression of the gene of interest, leading to permanent expression following electroporation.

### Procedure to target internal tissues

1. Make the injection box: boil 1.2% agarose in embryo medium, pour in Petri dishes and overlay with the injection molds; let set then remove the mold and cover with embryo medium;
2. Anesthetize 24 hpf-old, dechorionated embryos in Tricaine-containing embryo medium;
3. Dispose the embryos into the transplantation holes;
4. Inject the DNA inside the brain (in the lumen if it is formed, otherwise between the cells), using a micropipette with a rather big opening;
5. Immediately transfer the injected embryos with a glass Pasteur pipette to the electroporation chamber filled with embryo medium (Hank's saline) (several embryos can be electroporated at once);
6. Connect the electric wires, and orient the embryos such that the "+" pole is on the side to be targeted
7. Apply current with the following settings: Mode: low voltage
   Voltage: 10-20 V
   Pulses length: 50 ms
   Number of pulses: 3-10
   Interval between pulses: 1s
8. Remove the embryos from the chamber and let them recover in embryo medium at 28.5°C until the desired stage of analysis.

### Procedure to target external tissues

1. Dechorionate the embryos;
2. Pipet them into the electropration chamber filled with embryo medium;
3. Pipet out the medium carefully and replace it with a 0.5-1 mg/ml DNA solution in embryo medium;
4. Proceed as above from step 6.

### Material needed

- Injection mold (for transplantation holes) and agarose to make injection box
- Microinjector
- Pipette puller, micropipettes

- Electroporator (Electro Square Porator $^{TM}$ ECM 830, BTX – Genetronix, Inc -)
- Electroporation cuvette with electrodes
- Plasmid DNA constructs, diluted at 0.5-1 □g/□l in Ambion $H_2O$ (e.g.: *pCMV-GFP*)
- Anesthetic: Tricaine (3-amino benzoic acidethylester, Sigma A-5040), 25x stock is 4 mg/ml Tricaine in 20 mM Tris-HCl pH 7.5; dilute in embryo medium for use

## References

Tawk et al., *Genesis* 32, 27-31 (2002)

## Buffers and solutions

### PBT

| | |
|---|---|
| PBS | 1x |
| Tween-20 | 0.1% |

### PTD

| | |
|---|---|
| PBS | 1x |
| Triton x100 | 0.3% |
| DMSO | 1% |

### PTDBN

| | |
|---|---|
| PTD | 1x |
| BSA | 2 mg/ml |
| Normal Goat Serum | 1% |

### Lysinated rhodamine dextran, fixable (Molecular Probes D-1817)

5% stock in $H_2O$

### Anesthetic: Tricaine (3-amino benzoic acidethylester, Sigma A-5040)

25x stock Tricaine

Tricaine 4 mg/ml

in 20 mM Tris-HCl pH 7.5

dilute in embryo medium for use

### 4% paraformaldehyde (PFA)

solution in PBS

# 5. HIGH THROUGHPUT SECREENING OF EXPRESSION PATTERNS

(Rebecca Quiring, Beate Wittbrodt, Jochen Wittbrodt)

## 5.1 PCR transcripts from a gridded cDNA library

The cDNA library is a liquid culture of 384 colonies/384-well plate; stored at –80°C

From the library plates, fresh replicas were made as follows:

1. upon total thawing of the plates at room temperature bacteria were transferred with a 384replicator into 65-70µl of LB (10% glycerol, 0.02g/AMP100ml)
2. plates were incubated at 37°C for 16h (without shaking)
3. original plates were re-frozen on dry ice (let freeze bottom to top)
4. 10µl PCR Master Mix was pre-pipetted into a 384well plate with a 12channnel pipettor
5. with a replicator the freshly grown bacteria were transferred into a 384-well plate containing the Master Mix; plates were covered with a silicon cover

PCR reaction:

| | | |
|---|---|---|
| 96°C | 3min | |
| 96°C | 10sec | } 30x |
| 70°C | 3min | |
| 72°C | 8min | |
| 18°C | hold | |

1µl of each PCR reaction was analysed on a 1.5% agarose gel (1xTAE )

"PCR" plates were frozen at -20 °C

### Buffers and solutions

#### Master Mix for 400 10µl PCR reactions:

| | |
|---|---|
| 10xPerkin Elmer taq polymerase buffer | 400 µl |
| 3/86 primer (100pm/µl) | 40 µl |
| M13fwd primer (100pm/µl) | 40 µl |
| taq polymerase (Quiagen) | 40 µl |
| dNTPs | 40 µl |
| $H_2O$ | 3440 µl |

#### 10x Perkin Elmer buffer:

| | |
|---|---|
| $MgCl_2$ | 15mM |
| Tris-HCl pH 8.3 | 100mM |
| KCl | 500mM |

(no gelatine)

dNTPs

dNTP Mixture 2.5mM each (from TaKaRa or other brand)

## Material needed

- 384 replicator: NUNC
- 384well plate: NUNC; Kat.Nr.242765 clear (non treated) w/lid , sterile,PS, 120µl well
- PCR-Mikrotestplatte 384:LA-BIO-MED Kat.Nr.4028-0384 PP, ns,RNAse/DNAse-free (25µl well)
- Silikonmatte PCR384: LA-BIO-MED; Kat.Nr.1000-1384,Sil., ns, RNAse/DNAse-free
- Gradient cycler: MJ research, model: PTC-200

---

# 5.2 Digoxigenine labelled RNA probes of PCR transcripts

1. pre-pipette 9,5 µl Master Mix into 96wells of 384 plate (use e.g. Eppendorf pipettor)
2. thaw 384well plate with PCR transcripts from library clones
3. add 0.5 µl of each PCR reaction with 12channel pipettor to prepipetted Master Mix
4. incubate at 37°C for 5h
5. test a few samples (1 in 10) on 1% agarose gel (1 µl from the 10 µl reaction with 1-2 µl 2xRNA loading buffer)
6. freeze until purification

## List of material needed:

- RNAguard=RNAse inhibitor (between29100 and 35800U/ml), Amersham Pharmacia
- Thermostable inorganic Pyrophosphatase , (2000U/ml), New England BioLabs
- rNTP-Mixs: ATP, CTP, GTP 15,4mM, UTP 10mM, Roche
- Digoxigenin-11- UTP, Roche
- SP6 Polymerase (20U/µl), Roche

## Buffers and solutions:

### 2xRNA-loading buffer

| | |
|---|---|
| deonized formamide | 100 ml |
| EDTA, 0.5M | 2 ml |
| xylene cyanol | 0.1% |
| bromophenol blue | 0.1% |

### Master Mix for 100 x transcripts:

| | |
|---|---|
| $H_2O$ | 580 µl |
| 10x transcription buffer | 100 µl |
| RNAguard | 50µl |
| 40mM $MgCl_2$ | 50 µl |
| Thermostable inorganic Pyrophosphatase | 20 µl |
| RNTPs | 65 µl |
| Dig UTP | 35 µl |
| SP6 Polymerase | 100 µl |

---

# 5.3 Purification of RNA-dig probes

Use Quiaquick PCR purification kit 96 with the QIAvac 96 according to manufactures instructions except

1. add 350 µl of buffer RLT ( "RNAesy Kit" ) to the QIAquick 96 Plate
2. add the 10µl RNA dig labelled probe (using 12channnel pipettor)
3. add 250µl ETOH with "eppendorf research pro" pipettor set on "pip" with "rinse" option
4. switch on vacuum source- pull liquid through-turn off vacuum
   continue according to manual:

5. add 900µl of Buffer PE to each well
6. switch on vacuum source- pull liquid through-turn off vacuum
7. repeat last steps: "add 900µl PE…" etc.
8. after Buffer PE in all wells has been drawn through, apply maximum vacuum for an additional 10min to dry the membrane
9. switch off vacuum source, ventilate QIAvac 96 slowly. Lift top plate from the base (not 96 plate from the top plate), vigorously rap the top plate on a stack of absorbent paper (until no drops come out, and blot the nozzles of the plate with clean absorbent paper
10. replace waste tray with collection microtube rack (add 96plate back)
11. elute with 80µl of buffer EB added to the center of each well
12. let stand for 1min!!!
13. switch on vacuum source for 5min
14. test a few samples (1 in 10) on 1% agarose gel (4µl with 4µl 2xRNA loading buffer)
15. add 150µl of Hybridization Mix

## Material needed

- Quiaquick PCR purification kit 96
- QIAvac 96
- Ethanol
- "eppendorf research pro" pipettor
- 1% agarose

## Buffers and solutions

### Hybridization Mix:

| | |
|---|---|
| formamide (Fluka, ultra pure) | 50% |
| SSC | 5x |
| Heparin | 50 µg/ml |
| Tween20 | 0.1% |
| torula RNA | 5 mg/ml |

store at 20°C

---

# 5.4 Preparation of embryos

1. separate embryos (only for medaka embryos) by rolling them gently in the petri dish or on a sheet of Whatman-paper
2. transfer separated embryos to a 25 ml glass vial and fix for 4 hrs at room temperature in 4% PFA/2xPTW by vigorously rocking on a shaker
3. transfer embryos to a small petri dish and remove chorion with two forceps
4. transfer embryos back to glass vial and wash 4 x 5 min in 20 ml PTW
5. wash 5 min at room temperature in 100% MeOH
6. replace MeOH and store embryos at least over night at -20°C

Comments: MeOH treatment enhances probe penetration, a longer storage of embryos at -20°C in MeOH is advantageous.

All steps are performed at RT on a shaker except the digestion itself; volumes of solutions used per well are 3-5ml. Transfer embryos of different stages to a 6-well cell culture dish (or 3cm petridishes)- stages: 4,5 or 6 day into separate 3cm petridish

1. rehydrate 5min in 75% MeOH/PTW
2. rehydrate 5min in 50% MeOH/PTW
3. rehydrate 5min in 25% MeOH /PTW
4. rinse 2x5min each in PTW

5. prepare ProteinaseK (10□g/ml in PTW -use prepared stock (20mg/ml) 1:2000= 10□l/20ml) without shaking- time according to stage of embryos

| | |
|---|---|
| stage 18 | 7min |
| stage23 | 9min |
| 4-5days | 1h-1h15min |
| 6days | 1h+20min-1h30min |

6. prepare freshly 2mg/ml glycine/PTW (0.1g/50ml or 0.04g/20ml PTW)
7. prepare 4% PFA/PTW
8. rinse 2x shortly in glycine/PTW
9. fix in 4% PFA/PTW for 20min
10. wash 5x 5min in PTW
11. use immediately or put to MeOH and store at –20 (rehydrate prior to loading the machine)

### Buffers and solutions

PTW (2xPTW):

1x PBS (2xPBS), pH 7.5, add Tween20 to 0.1% and sterile filter (0.2 µm, nitrocellulose).

4% PFA: dissolve 16% paraformaldehyde in PBS by stirring and heating to 65°C, add dropwise 1 M NaOH until the solution gets clear (check pH ≈ 7.5), cool to room temperature and store at 4°C (for up to 4 weeks; if Medaka embryos do not stay clear but turn "milky" in MeOH prepare fresh 16% PFA stock). Mix with 3 volumes of 2xPTW just prior to use.

Use for early stages ( ≥16) use 1 1/2 x PTW + PFA and later stages (□17) 2x PTW + PFA for the fixation!

ProteinaseK: prepare a stock solution of 20 mg/ml and store frozen aliquots at -20°C, dilute 1:2000 just prior to use to a final concentration of 10 µg/ml in PTW

---

## 5.5 Setup of the INTAVIS insituPro robot

1. put fresh rinsing reagents into the insitu robot
2. put fresh empty in situ tubes into machine
3. fill machine completely to prevent evaporation
4. cleaning run with water and $H_2O_2$

5. after cleaning run remove in situ tubes form the robot and put them into loading rack filled with $dH_2O$ (pipet tip box)
6. fill in situ tubes with PTW
7. distribute equilibrated embryos with wide-bore eppendorf tip to approx 5-10 of each stage in each in situ tube
8. put in situ tubes with embryos (lid on) into the robot
9. put tubes with setup reagents into robot

## Buffers for INTAVIS insituPro robot

1. add buffers to tubes and tubes into slots according to the letters marked on them

| tubes: | | for 48x in situs: |
|---|---|---|
| **C** | Hybridization mix | 20ml |
| **D** | 50% formamide/2xSSCT | 20ml |
| **E** | 2xSSCT | 20ml |
| **F** | 0.2xSSCT | 20ml |
| **L** | blocking buffer: | 10ml |
| | sheep serum 5% (500µl/10ml) | |
| | PTW | |
| **M** | preabsorbed anti-dig-UTP antibody | 9ml |
| **A** | PTW, add additional 0.1%Tween20 | 100ml |
| **K** | staining buffer=SB: | 150 ml |
| | TrisCl, pH 9.5 100mM | |
| | NaCl 100mM | |
| | $MgCl_2$ 50mM | |
| | Tweeen20 0.1% | |

## Material needed

- formamide
- sheep serum (Sigma s-2263)
- ant-dig-UTP antibody (preabsorbed , see below)
- Tween 20
- 4xSSCT**:** dilute 20xSSC to 4xSSC and add Tween20 to 0.1%

---

# 5.6 Pre absorption of antibodies

embryos need not to be dechorionated

1. fix embryos in 4% PFA/PTW 4 hrs at room temperature or at 4°C overnight
2. wash 4 x 5 min in PTW
3. store embryos in 100% MeOH at -20°C
4. take 1 ml embryos and rehydrate by washing 3 x 5 min in PTW
5. transfer embryos to 2 ml Eppendorf tube and homogenize in PTW with a pestle
6. adjust volume to 1 ml PTW
7. add 10 µl antibody (final: 1:100) and incubate by shaking at least overnight or store for several days at 4°C
8. spin down embryonic debris and sterile filter supernatant (cellulose acetate, 0.2 µm)
9. resuspend embryonic debris in PTW, spin and sterile filter again.
10. combine filtered antibody solutions, add 0.5 ml sheep serum and 200 µl 3% Sodium azide, and fill up with PTW to 20 ml (final: 1:2000)

11. store pre absorbed antibody at 4°C, it will be stable for 2-3 month

Comments: pre absorbed antibodies can be reused twice, zebrafish or medaka pre absorbed antibodies work well also for staining of *Drosophila* embryos.

---

## 5.7 Adding the RNA probes to the insituPro robot

1. use 190µl Hybridization Mix + 10µl dig probe in 8-tube strips (PCR strips) (alternatively use 5µldig probe +195µl HybMix)
2. put cap on strips and heat to 80 °C for 10min in PCR machine to denature the probe
3. remove caps and put tube strips containing the denatured probes into robot

---

## 5.8 Running of the program

The program has been optimized to run efficiently with strong staining for approx. 20 hrs. It will pause automatically and will wait for the addition of freshly prepared staining buffer (see above) in slot K. Push any key to continue.

---

## 5.9 Staining

### List of material needed

Staining buffer (see above)
BCIP (Roche)
NBT (Roche)

1. take columns out of robot and stand in water filled transfer racks/ boxes (e.g.empty boxes from Art 200 pipette tips)
2. transfer embryos into 24well dish by filling columns to the rim with SB (stainig buffer) then tilting them into the well already containing SB and make fluids surfaces contact
3. dissolve 4.5µl NBT per ml SB (final 337.5 µg/ml) and 3.5 µl per ml SB (final 175 µg/ml) and add to embryos after sucking off SB
4. stain in the dark without shaking up to 12h, monitor staining after 30 minutes, renew staining solution if necessary
5. to stop reaction: wash 3x 1min in PTW
6. store in PTW+ ~1% PFA (3-5ml of 16% PFA for 50ml of PTW)

## 5.10 Cleaning of the incubation columns

after each hybridization procedure according to Intavis instructions :

1. disassemble the columns, rinse the individual parts with water
2. store them for about 12 hours in a solution of 0.1M NaOH
3. rinse the parts again
4. place the parts in a container filled with ethanol
5. finally dry them in a drying cupboard for 1 hour at 50 °C maximum

### References

Hauptmann G. and Gerster T. (1994). Two color whole-mount in situ hybridization to vertebrate and *Drosophila* embryos. *Trends Genet.* **10**: 266

# 6. WHOLE MOUNT RNA OR PROTEIN DETECTION

## 6.1 (Additional protocol) Whole mount *in situ* hybridization on zebrafish embryos using digoxigenin probes

(Alexander Schier)

### Probe synthesis

This is essentially according to the method recommended by Roche Mannheim. Cut 10 ug of plasmid DNA with appropriate enzyme in 100 µl. Phenol, phenol/chloroform, chloroform and ethanol precipitate. Dissolve in 20 µl dd$H_2O$.

1. Mix together 2 µl of linearised plasmid (1 µg of insert DNA); 2 µl of 10x transcription buffer (400 mM Tris-HCl pH 8.0; 60 mM MgC12; 100 mM dithiothreitol; 20mM spermidine; 100 mM NaCl; RNase inhibitor 1 unit/µl); 2 µl of 10x nucleotide mix (10 mM each ATP, GTP, CTP; 6.5 mM UTP; with either 3.5 mM digoxigenin-UTP); 1 µl (20 units) of RNase inhibitor. Add 11 µl water to give a final reaction volume of 20 µl and 2 µl (40 units) of the appropriate T7, T3 or SP6 RNA polymerase. Incubate the mixture for 2 hour at 37°C.

2. Add 2 µl (40 units) of DNase I and incubate at 37°C for 30 min. to remove the plasmid DNA.

3. Stop the reaction (optional) by adding 2 µl EDTA (0.2 M pH 8.0) and precipitate the RNA with 2.5 µl 4 M LiCl, and 75 µl prechilled ethanol for 30 min on dry ice.

4. Spin down the pellet and redissolve in 100 µl of RNAse-free water. The probe can be checked by running 5 µl (should be about 500 ng) on a minigel. (As a "standard": 5 µl from tube 5 in kit correspond to 500 ng RNA). Probes are stored in aliquots at -20°C. Safest to store them in Hyb+ solution. We have had success with probes of 0.5-2 kb and signals are stronger if they are not hydrolyzed prior to use (requires proteins K step!). I did not see any improvement by removing the nucleotides e.g. by NucTrap columns.

### Preparation of embryos

Embryos are mixed during most incubations by pipetting up and down incubation media or by turning on wheel. Heavy mixing or media/air interface contacts lead to damage of embryos.

1. Embryos younger than 20 somites are best left non-dechorionated and fixed overnight (or longer) in 4% paraformaldehyde/PBS at 4°C. Wash twice 5 min in PBS or PBT and dechorionate manually.
2. After a few minutes of equilibration with 4-5 changes of methanol the embryos are stored at -20°C in glass specimen vials or Eppendorf tubes. (Embryos can be stored in this way for several months.)

3. Rehydrate fixed embryos by soaking for 5 min each in 75% methanol + 25% PBT (lxPBS, 0.1% Tween 20); 50% methanol + 50% PBT; 25% methanol + 75% PBT. Young embryos (before 1S) seem to be rather fragile during rehydration!! Then 4x 5 min in 100% PBT.
4. Incubate embryos in proteinase K at room temperature (10 µg/ml PBT. I use -20$^{o}$C aliquots of 1mg/ml proteinase K in PBT. Dilute 100x before use). 5-10 min for pre 1S, 10-15 min pre 16h, 15-20 min post 16h. This step ideally should be titrated. Wash 2x PBT.
5. Refix embryos in 4% paraformaldehyde in 1x PBS for 20 min. at room temperature.
6. Rinse 5x 5 min. in PBT.

## Hybridization

1. Prehybridize embryos in hybridization buffer (50% formamide, 5xSSC (pH 7.0), 500 µg/ml torula (yeast) RNA, 50 µg/ml heparin, 0.1% Tween 20, 9mM citric acid to pH 6.0-6.5) for at least 1 hour (preferably for 4-5 h) at hybridization temperature (60-70°C). The hybridization temperature is dependent on the probe but we have obtained good signals with all our probes at 65-70°C in 50% formamide. Zebrafish *krox-20* and pax-b also work very well at 70°C in 65% formamide.
2. Replace prehybridization buffer with hybridization buffer containing probe. We use 150 ng digoxigenin riboprobe in 200 µl hybridization solution (preheated) as a starting point.
3. Incubate overnight at 65-70°C in a 1.5 ml microfuge tube in a heating block or hybridization oven or water bath. Probes are stable in hyb and can be reused several times.
4. Washes are all done at the hybridization temperature with preheated solutions. Mix after 5 min by turning tube. 10 min 75% hyb + 25% 2x SSC; 10 min 50% hyb + 50% 2x SSC; 10 min 25% hyb + 75% 2x SSC; 10 min 100% 2x SSC.
5. Wash embryos 2x 30 min. in 0.2x SSC at 70°C.
6. Perform the following washes at room temperature: 5 min 75% 0.2x SSC + 25% PBT; 5 min 50% 0.2x SSC + 50% PBT; 5 min 25% 0.2x SSC + 75% PBT; 5 min 100% PBT.

## Preabsorption of the anti-digoxigenin antibodies.

The antibody must be pre absorbed against zebrafish embryos or adult zebrafish acetone powder (Grind up several hundred day 2 embryos in minimal volume of egg water or freeze adult in liquid nitrogen and grind up. Add 4x vol. cold acetone, leave on ice for 30 min. Spin down 10k 10 min. Discard S/N and wash again in acetone. Spin down, discard S/N and let embryo powder dry on filter paper. Pulverize and keep at 0.2% in 2 mg/ml BSA in PBT) Add 1 µl(0.75 units) antibody to 400 µl of 0.2% w/v zebrafish powder in 2 mg/ml BSA in PBT. Rotate sideways for at least 1 hour at room temperature, then spin in a microfuge before diluting to the desired concentration (1:2000). The diluted antibody is stable under these conditions at 4°C and we have reused it up to three times. Anti-Fluorescein antibody coupled to HRP is used at a final 1:200 dilution of 0.15u/µl stock(i.e. twice as concentrated as anti-dig).

## Antibody incubation and staining

1. Block with 2 mg/ml BSA, 5% sheep serum in PBT for a minimum of 60 min.
2. ncubate for 2 h at RT. or o/n at 4°C in 200 µl of a 1/2000 dilution (0.375 units/ml) of preabsorbed sheep anti-digoxygenin-alkaline phosphatase conjugated Fab fragments or 1/200 (0.75 units/ml) anti-fluorescein-HRP in 2mg/ml BSA PBT.
3. Wash for at least 2 h in 2mg/ml BSA PBT with 8 solution changes.
4. Equilibrate 3x 5 min in freshly made NTMT buffer (0.1 M Tris-HCl pH 9.5; 50 mM MgC12; 0.1 M NaCl; 0.1% Tween 20). Stain with X-phosphate/NBT on shaker in dark (4.5 µl of 75 mg/ml NBT in

70% dimethylformamide and 3.5 µl of 50 mg/ml X-phosphate in dimethylformamide added to 1 ml of NTMT buffer). Take care that embryos do not stick together.

5. Stop reaction with washes in PBT. krox-20 comes up within minutes, shh and gsc take longer.

## Clearing and embedding

1. Dehydrate 2x 5 min in methanol
2. Clear in benzylbenzoate/benzylalcohol = 2:1. Precipitate will slowly dissolve (within few days) in this solution. Keep in fridge. Mount in Permount and take pictures immediately after clearing. Embryos are very fragile.

Special Note:

no DEPC treated water used, all embryo incubation buffers (also where not specifically mentioned) except paraformaldehyde contain 0.1% Tween 20

## Reagents

| | |
|---|---|
| Dig RNA labeling mixture (10x) for 20 RNA samples | Roche 1277 073 |
| Anti-Digoxygenin Fab fragment - alkaline phosphatase; 150 u | Roche 1093 274 |
| T3 RNA polymerase; 1000 u + 10x buffer | Roche 1031 163 |
| T7 RNA polymerase; 1000 u + 10x buffer | Roche 881 767 |
| RNase inhibitor; 2000 u | Roche 799 017 |
| DNase I; RNase free 10000 u | Roche 776 785 |
| Proteinase K; 100 mg | Roche 745 723 |

1xPBS (2 l): 16 g NaCl; 0.4 g KCl; 2.88 g $Na_2HPO_4$; 0.48 g $KH_2PO_4$ pH 7.4

1xPBT: 1x PBS + 0.1% Tween 20

4% paraformaldehyde/PBS: 4 g in 100 ml PBS, dissolve at 68°C, add a few drops of 5N NaOH, check pH 7.4. freeze -20°C

20x SSC: 1 l: 175.3 g NaCl; 88.2 g sodium citrate pH 7.0

## Hyb

50% formamide
5xSSC
500 µg/ml torula (yeast) RNA Sigma 100g R-6625
50 µg/ml heparin
0.1% Tween 20
9 mM citric acid
(final pH is 6.0-6.5 !)
BSA: 50g bovine albumin Sigma A-8022

## Staining Buffer

0.1 M Tris HCl pH 9.5; 0.1 M NaCl; 0.05 M $MgCl_2$; 0.1% Tween 20

500 ml: 6.05 g Trizma; 2.92 g NaCl; 25 ml 1M $MgCl_2$; 5 ml 10% Tween 20; ca. 1ml 2M HCl to pH 9.5

### Substrate solution

50 ml staining buffer + 225 µl NBT (75 mg/ml NBT in 70% dimethylformamide) + 175 µl X-phosphate (50 mg/ml X-phosphate in dimethylformamide)

---

## 6.2 (Additional Protocol) Antibody Staining in zebrafish

(Schier, Neuhauss, Schulte-Merker, Solnica-Krezel, Holder, Hatta)

(Extensive washing protocol, found to reduce background significantly for HNK-1 staining). Good for 24 hours and older. Gastrula stage embryos: might have to omit aceton permeabilization

*day 1*

1. Fix in 4% Paraformaldehyde in PBS 1h at RT.
2. wash 4 times 5 minutes each in PBT pH 7.4
3. wash 1 time 5 minutes in $ddH_2O$
4. permeabilze for 7 minutes in aceton -20°C
5. wash 1 time in $H_2O$
6. wash 1 time in PBDT (0.1% Tween 20, 1% DMSO in PBS)
7. block in PBDT-10%NGS (PBDT plus 10% normal goat serum) for 100 minutes or more
8. 1st antibody in PBDT-1%NGS o/n at 4°C on shaker (mab culture supernatants 1:1 to 1:50 ; ascites 1:1000 to 1:10000 ; rabbit etc. serum 1:100 to 1:5000; optimal dilution needs to be determined for each antibody)

*day 2*

1. wash 2 times 1 minute and 6 times whole day in PBDT-1% NGS+0.1M NaCl, last wash in PBDT-1%NGS
2. 2nd antibody IgM (HNK-1) o/n 4°C at dilution of 1:1000 Vectastain Elite (in flies I used 1:500) in PBDT-1%NGS.

*day 3*

1. wash 2 times 1 minute and 5 times whole day (optional: and o/n in PBDT-0.1% NGS+0.1M NaCl)

*day 4*

1. prepare ABC complex by incubating during last wash (30 min) 10 µl A + 10 µl B in 1 ml PBDT.
2. wash 2x 30 min in PBDT.
3. incubate embryos in ABCPBDT for 30-60 minutes
4. wash 2 times 1 minute and 3 times 20 minutes in PBDT

for brown staining:

5. wash in 100mM TrisCl buffer/Tween (10 ml 1M TrisCl pH 7.5 + 90 dd$H_2O$ + 100 µl 10% Tween20) for 15 minutes (pH. 7.2 with acetic acid)
6. stain with DAB/TrisCl solution (8.5 ml d$H_2O$ + 1 ml 1M TrisCl pH 7.5 , 0.1% Tween20 + 500 µl 10mg/ml DAB in Tris). 10-30 min preincubation, add 20 µl of 0.3% $H_2O2$, follow under scope.
7. stop by washing in Tris/Tween and PBDT.
8. dehydrate in methanol, can leave o/n, clear in benzylbenzoate/benzylalcohol = 2/1, view in this or mount in Permount

## 6.3 (Additional protocol) Two color whole-mount in situ hybridization in medaka/zebrafish

(M. Carl and J. Wittbrodt)

To simultaneously detect two messages in one embryo by whole mount in situ hybridization two different RNA probes labeled with Digoxigenin-UTP and Fluoresceine-UTP respectively are hybridized simultaneously.

Detection is performed in two consecutive rounds.

The two colors used are Fast Red (weaker substrate) and NBT/BCIP (blue).

For best results the more abundant mRNA should be detected with a Fluoresceine-UTP labeled probe in the first round of detection using Fast Red as a substrate.

Outline:

- Fix embryos
- Prepare RNA probe
- Dechrionate, wash and dehydrate embryos
- Check probe
- Rehydrate, proteinase K (or heat) treat, postfix and wash embryos
- Prehybridize and hybridize embryos
- Wash embryos
- Blocking and 1st AB incubation
- Wash embryos
- Detection
- 1st antibody removal
- 2nd AB incubation
- Wash embryos
- 2nd probe detection
- Fix, mount and take pictures

### Fixation and storage of embryos

**Reagents:**

PTW (2xPTW): 1x PBS (2xPBS), pH 7.5, add Tween20 to 0.1% and sterile filter (0.2 µm, nitrocellulose).

4% PFA: dissolve 16% paraformaldehyde in PBS by stirring and heating to 65°C, add dropwise 1 M NaOH until the solution gets clear (check pH ≈ 7.5), cool to room temperature and store at 4°C (for up to 4 weeks; if Medaka embryos do not stay clear but turn "milky" in MeOH prepare fresh 16% PFA stock). Mix with 3 volumes of 2xPTW just prior to use.

1. Separate embryos (only for medaka embryos) by rolling them gently in the petri dish or on a sheet of Whatman-paper
2. Transfer separated embryos to a 25 ml glass vial and fix for 4 hrs at room temperature in 4% PFA/2xPTW by vigorously rocking on a shaker
3. Transfer embryos to a small petri dish and remove chorion with two forceps
4. Transfer embryos back to glass vial and wash 4 x 5 min in 20 ml PTW
5. Wash 5 min at room temperature in 100% MeOH
6. Replace MeOH and store embryos at least over night at -20°C

Comments: MeOH treatment enhances probe penetration, a longer storage of embryos at -20°C in MeOH is advantageous. Set up fixed embryo stocks during periods of good egg laying.

## RNA probe preparation

### Reagents:

NTP-Mix: ATP, CTP, GTP 15.4 mM each, UTP 10.0 mM (all Roche)
Digoxigenin-11-UTP 10 mM (Roche)
Fluorescein-12-UTP 10 mM (Roche)
RNasin 20-40 U/µl (Promega, Pharmacia)
T7-/SP6-/T3-RNA-Polymerase 20 U/µl (Roche)
5xTranscriptionbuffer (Stratagene)
DNaseI RNase-free 10U/µl (Roche)
STE: 100 mM NaCl/20 mM TrisCl, pH 7.5/10 mM EDTA
RNeasy (Qiagen)
Hybridization Mix-$H_2O$

| | stock | HYB-$H_2O$ |
|---|---|---|
| Formamide | 100 % | 5 ml |
| SSC | 20 x | 2.5 ml |
| Heparin | 50 mg/ml | 10 µl |
| Torula-RNA (Sigma) | solid | 50 mg |
| Tween20, | 10 % | 10 µl |

Nylon-membrane (HybondN, Amersham)
PI: 100 mM TrisCl, pH 7.5/150 mM NaCl
PII: PBS/0.1% TritonX-100
PIII: 100 mM TrisCl, pH 9.5/100 mM NaCl/50 mM $MgCl_2$
Anti-Digoxigenin-Fab fragments/Anti-Fluoresceine-Fab fragments (Roche)

BCIP (Roche): 50 mg/ml in 100% DMF

NBT (Roche): 75 mg/ml in 70% DMF/$H_2O$

1. Linearize 10 µg of template with a suitable enzyme allowing as transcription (blunt or 5-prime overhang should be preferred to avoid snap back effects)
2. Purify template from enzyme and digestion buffer (QiaQuick nucleotide removal kit, Qiagen)
3. Control for a complete digest on an agarose gel
4. Add in the following order to a total volume of 20 µl:

| | |
|---|---|
| linearized template | 1 µg |
| 100 mM DTT | 2 µl |
| NTP-Mix | 1.3 µl |
| 10 mM Dig-UTP/Fluorescein-UTP | 0.7 µl |
| Rnasin | 0.5 µl |
| 5xTranscriptionbuffer | 4 µl |
| $H_2O$ | ad 19 µl |
| RNA-Polymerase | 1 µl |

5. Incubate for 2 hrs at 37°C
6. Add 1 µl DNaseI and incubate for another 15 min at 37°C
7. Add 50 µl $H_2O$
8. Purify RNA using the Quiagen RNeasy kit (fast, relatively cheap)
9. Elute the RNA from the column with 2x25 µl elution buffer
10. Take an aliquot of 1-2 µl and load in formamide loading buffer onto a TAE agarose gel (prepared from RNase free stocks) followed by 1 hr blotting to a nylon-membrane (HybondN, Amersham)
11. Incorporation of Dig-/Fluorescein-UTP is controlled by 1 µl aliquots of a dilution series (1:1, 1:10, 1:50, 1:100, 1:500) on a nylon-membrane (HybondN, Amersham)
12. Dried membranes are equilibrated for 1-2 min in PI
13. Block membrane for 30 min in PII by gentle shaking
14. Incubate membrane 30 min in 1:2000 dilution of antibody (Anti-Digoxigenin or Anti-Fluoresceine respectively) in PII (≥0.2 ml/$cm^2$)
15. Wash twice 15 min each in PI (≥1 ml/$cm^2$)
16. Incubate membrane 2 min in PIII
17. Stain membrane for 5 min in 4.5 µl/ml NBT and 3.5 µl/ml BCIP in PIII
18. Wash 1-2 min in PII and take picture
    A good probe should give a signal at 1:500 dilution after 2-3 min
19. Dilute probe in 150 µl Hyb-$H_2O$ and store at -20°C

Comments:

As a rule of the thumb 1 µl of the probe in Hyb-mix will give a good staining.

Probes from 0.4 - 2.5 kb were used successfully with this protocol, short probes below 0.4 kb will generate much background although this varies with the specificity of each probe and may be worth being tried.

Hydrolysis of the probe might be required in a few exceptional cases.

## Proteinase digestion and postfixation

Reagents:

ProteinaseK: prepare a stock solution of 20 mg/ml and store frozen aliquots at -20°C, dilute 1:2000 just prior to use to a final concentration of 10 µg/ml in PTW

4% PFA see above

All steps are performed at room temperature on a shaker except the digestion itself, volumes of solutions used per well are 3-5 ml

1. Transfer embryos of different stages to a 6-well cell culture dish
2. Rehydrate 5 min in 75% MeOH/PTW
3. Rehydrate 5 min in 50% MeOH/PTW
4. Rehydrate 5 min in 25% MeOH/PTW
5. Rinse 2 x 5 min each in PTW
6. Digest with ProteinaseK (10 µg/ml in PTW) without shaking for several minutes depending on the stage of the embryos
7. Rinse 2 x shortly in freshly prepared 2 mg/ml glycine/PTW
8. Fix in 4% PFA/PTW for 20 min
9. Wash 5 x 5 min in PTW

Comments: The proteinaseK-digestion is a critical step of this protocol and will substantially influence the quality of the whole mount in situ, thus, times of digestion have to be optimized for each problem. The following list of digestion times worked well in our hands and can be used as guideline

| stage* | time of proteinaseK digestion |
|---|---|
| 1 - 13 | 1 - 2 min |
| 14 - 16 | 3 - 4 min |
| 17 - 20 | 5 min |
| 21 - 24 | 7 min |
| 25 - 30 | 10 - 15 min |
| > 30 | > 15 min |

* stages are referred to Yamamoto, Medaka (Killifish), Biology and Strains, Keigaku Publishing Company, Tokyo, Japan

## Embryo pretreatment by heat denaturation

(as an alternative to ProteinaseK treatment)

1. rehydrate embryos in the glass scintillation vial as outlined above. have the embryos in 6 ml 1xPTW
5. heat waterbath to 100°C, when boiling, turn it off
6. put glass scintillation vial into a styrofoam-float, gently agitate embryos and incubate in the waterbath for 5 min (gentle agitation every 1.5 min during the incubation prevents embryos from sticking together).
7. chill on ice to cool down to RT (shake gently)
8. ready

# Hybridization

## Buffers and Solutions

### Heparin

make a stock of 50 mg/ml in $H_2O$, store at -20°C

### Hybridization Mix:

50% formamide (Fluka, ultra pure), 5xSSC, 50 µg/ml heparin, 0.1%Tween20, 5 mg/ml torula RNA, store at -20°C,

for 50 ml of Hyb-Mix:

| | stock | Hyb-mix |
|---|---|---|
| Formamide | 100 % | 25 ml |
| SSC | 20 x | 12.5 ml |
| Heparin | 50 mg/m | l50 µl |
| Torula-RNA (Sigma) | solid | 250 mg |
| Tween20 | 10 % | 500 µl |
| $H_2O$ | | ad 50 ml |

All steps are performed in a water bath preheated to 65°C

1. Transfer embryos to 2 ml Eppendorf tubes
2. Prehybridize 1-2 hrs in 1 ml Hyb-Mix at 65°C
3. Denature probe (1-5 µl/100µl Hyb-Mix, as a rule of the thumb 1 µl of the probe will give a good staining) in 100 µl of Hyb-Mix (use 200 µl when larger amounts of embryos are hybridized) for 10 min at 80°C
4. Remove prehybridization solution leaving embryos slightly covered to avoid their destruction, the embryos are very sensitive at 65°C
5. Quickly add hybridization probe, mix gently and hybridize at 65°C overnight

Comments: The right amount of probe has to be evaluated for each newly prepared probe, usually 1 µl of a tested probe per 100 µl Hyb-Mix works well.

## Washes

### Reagents:

4xSSCT: dilute 20xSSC to 4xSSC and add Tween20 to 0.1%

All steps are performed in a water bath, all wash solutions are prewarmed to 65°C

1. Wash embryos 2 x 30 min in 2 ml 50% formamide/2xSSCT at 65°C
2. Wash embryos 15 min in 2 ml 2xSSCT at 65°C
3. Wash embryos 2 x each 30 min in 2 ml 0.2xSSCT at 65°C

## Detection

### Reagents:

- Fast Red tablets: (Roche)
- BCIP (Roche): 50 mg/ml in 100% DMF
- NBT (Roche): 75 mg/ml in 70% DMF/$H_2O$
- SB: 100 mM TrisCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1%Tween20
- for 50 ml of SB add:

| | stock | 1xSB |
|---|---|---|
| TrisCl, pH 9.5, | 1 M | 2.5 ml |
| NaCl, | 5 M | 1.0 ml |
| $MgCl_2$, | 1 M | 2.5 ml |
| Tween20 | 10 % | 500 µl |
| $H_2O$ | | ad 50 ml |

RSB: 0.1 M TrisCl, pH 8.2, 0.1% Tween 20

### Primary staining

All steps are performed on a shaker except the staining reactions

1. Block embryos 1(-2) hrs with 1-2 ml of 5% sheep serum/PTW at room temperature
2. Incubate embryos for 1(-2) hrs in 200 µl pre absorbed anti-Fluorescein-AP $F_{ab}$ fragments at a 1 : 2000 dilution in PTW
3. Transfer embryos to a 6-well dish and wash 6 x 10 min while shaking in PTW at room temperature (one wash can be performed at 4°C overnight)
4. Equilibrate 2 x 5 min in 0.1 M TrisCl, pH 8.2/0.1%Tween
5. Dissolve Fast Red tablets in RSB (2 ml/tablet) and sterile filter (0.2 µm) to remove particles
6. Add staining solution to embryos and stain in the dark without shaking for up to 48 hrs, replace staining solution several times
7. Wash 3 x 5 min in PTW
8. If no second staining is required mount embryos

### Removal of antibody

All steps are performed on a shaker at room temperature

1. Incubate embryos 2 x 10 min in 0.1 M glycine/HCl, pH 2.2/0.1%Tween
2. Wash 4 x 5 min in PTW

### Secondary staining

All steps are performed at room temperature on a shaker except the staining reaction

1. Transfer embryos to a 2 ml Eppendorf tube
2. Incubate embryos for 2 hrs in 200 µl pre absorbed anti-Digoxigenin-AP $F_{ab}$ fragments at a 1:2000 dilution in PTW
3. Transfer embryos to a 6-well dish and wash 6 x 10 min in PTW while shaking
4. Equilibrate embryos 2 x 5 min in SB
5. Dissolve 4.5 µl NBT (final: 337.5 µg|ml) and 3.5 µl BCIP (final 175 µg/ml) per ml SB and add to embryos
6. Stain up to 48 hrs in the dark without shaking

7. Wash 3 x 5 min each in PTW

Comments: embryos in PTW can be restained after equilibration in SB and application of staining solution, stained embryos can be stored in PTW at 4°C for several days up to two weeks, which will reduce the background

8. Optionally refix embryos in PFA/PTW and wash 3x in PTW before mounting

## Mounting

1. Separate embryos from yolk in PTW
2. Transfer embryos to 87% glycerol
3. Leave in 87% glycerol at least overnight for complete equilibration
4. Mount in 87% glycerol in viewing chamber and take pictures

Comments: Glue one or two small coverslips respectively on a microscope slide (with super glue) to prepare a viewing chamber.

Additional hints

## Preabsorption of antibodies

Embryos need not to be dechorionated

1. Fix embryos in 4% PFA/PTW 4 hrs at room temperature or at 4°C overnight
2. Wash 4 x 5 min in PTW
3. Store embryos in 100% MeOH at -20°C
4. Take 1 ml embryos and rehydrate by washing 3 x 5 min in PTW
5. Transfer embryos to 2 ml Eppendorf tube and homogenize in PTW with a pestle
6. Adjust volume to 1 ml PTW
7. Add 10 µl antibody (final: 1:100) and incubate by shaking at least overnight or store for several days at 4°C
8. Spin down embryonic debris and sterile filter supernatant (cellulose acetate, 0.2 µm)
9. Resuspend embryonic debris in PTW, spin and sterile filter again.
10. Combine filtered antibody solutions and fill up with PTW to 20 ml (final: 1:2000)
11. Store pre absorbed antibody at 4°C, it will be stable for 2-3 month

Comments: Preabsorbed antibodies can be reused twice, zebrafish or medaka pre absorbed antibodies work well also for staining of *Drosophila* embryos.(?vice versa?)

Reference:

Hauptmann G. and Gerster T. (1994). Two color whole-mount in situ hybridization to vertebrate and *Drosophila* embryos. *Trends Genet.* 10: 266.

## 6.4 (Additional protocol) Immunohistochemistry in medaka/zebrafish

(R. Köster & J. Wittbrodt)

Immunohistochemistry can be combined with the in situ protocol if the antibody to be used is able to detect its epitope even after proteinase K treatment (e.g. AB to acetylated tubulin, anti isl-AB) This has to be determined experimentally. If the epitope is stable and abundant enough even 3 color detections (red, blue, green; immunodetection after two-color in situ hyb) are possible using NBT/BCIP, FastRed and naphtol-AS-GR-phosphate/Fast Blue BN (Roche multicolor detection set).

In every case in situ hybridization if performed first because there is probably no way to perform immunohistochemistry with RNase free reagents!!

Alternatively, if detection is to be performed on early stages of development (1 cell to 100% epiboly) in situ hybridization can be done omitting the proteinaseK step, which will allow to use a wider range of antibodies in the immunohistochemistry.

Instead of using secondary antibodies coupled to alkaline phosphatase of course peroxidase coupled antibodies can be used. We prefer phosphatase coupled antibodies because one can extend the staining time up to 48 hrs without dramatically increasing the background.

Outline:

- Fix embryos
- Dechrionate, wash and dehydrate embryos
- Rehydrate and wash embryos

Fixation, rehydration, of embryos is performed identical to
the protocol of whole-mount in situ hybridization

*(If double detection is performed do in situ hybridization first!!*
*- Remove AB from in situ detection at low pH)*

- Blocking and 1st AB incubation
- Wash embryos
- 2nd AB incubation
- Wash embryos
- Detection
- Fix, mount and take pictures

*If double detection is performed antibodies used in the in situ protocol have to be removed.*
*Removal of antibody*

All steps are performed on a shaker at room temperature
*- incubate embryos 2 x 10 min in 0.1 M glycine/HCl, pH 2.2/0.1%Tween*
*- wash 4 x 5 min in PTW*

## Application of Antibodies

### Reagents

MAb to acetylated tubulin, clone no. 6-11B-1, Sigma # T6793
mouse mAB to isl-1
Sheep Anti-Mouse Ig-AP, fab Fragments, Roche, # 1198 661
Sheep serum

All steps are performed at room temperature on a shaker

1. Transfer embryos to a 2 ml Eppendorf tube
2. Block embryos 1-2 hrs in 1-2 ml of 5% serum/PTW

3. Replace blocking solution by 500 µl of diluted (MAb to acetylated tubulin, 1:1000) first antibody in 5% serum/PTW and incubate for 2 hrs (alternatively at 4°C overnight)
4. Transfer embryos to 6-well dish
5. Wash 6 x 15 min in PTW
6. Transfer embryos to a fresh 2 ml Eppendorf tube and apply 200-500 µl of diluted (in our case: 1:200) pre absorbed secondary antibody coupled to alkaline phosphatase in 5% serum/PTW and incubate for 2 hrs (alternatively at 4°C overnight)
7. Transfer embryos to a 6-well dish
8. Wash 6 x 15 min in PTW

Comments:

The optimal dilution of primary and secondary antibody has to be established for each antibody to minimize the background and can range from 1:20 up to 1:5000. An established dilution for commercially available secondary antibodies will stay constant; the volume of wash solution in 6-well cell culture dish is 3-5 ml

## Detection

**Reagents:**

BCIP (Roche): 50 mg/ml in 100% DMF

NBT (Roche): 75 mg/ml in 70% DMF/$H_2O$

Fast Red tablets: (Roche)

RSB: 0.1 M TrisCl, pH 8.2, 0.1% Tween 20

SB: 100 mM TrisCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1%Tween20

for 50 ml of SB add:

| | stock | 1xSB | |
|---|---|---|---|
| TrisCl, pH 9.5 | | 1 M | 2.5 ml |
| NaCl | 5 M | 1.0 ml | |
| $MgCl_2$ | 1 M | 2.5 ml | |
| Tween20 | 10 % | 500 µl | |
| $H_2O$ | | ad 50 ml | |

All steps are performed at room temperature on a shaker except the staining reaction

Fast Red staining

1. Equilibrate embryos 2 x 5 min in 0.1 M TrisCl, pH 8.2/0.1%Tween
2. Dissolve Fast Red tablets in RSB (2 ml/tablet) and sterile filter (0.2 µm) to remove particles
3. Apply staining solution to embryos and stain without shaking in the dark up to 48 hrs, replace staining solution several times
4. To stop the staining reaction wash 3 x 5 min in PTW

NBT/BCIP staining

1. Equilibrate embryos 2 x 5 min in SB

2. Dissolve 4.5 µl NBT (final: 337.5 µg|ml) and 3.5 µl BCIP (final 175 µg/ml) per ml SB and apply to embryos
3. Stain up to 48 hrs in the dark without shaking
4. Wash 3 x 5 min in PTW

Comments: Embryos in PTW can be restained by equilibration in SB and application of staining solution, stained embryos can be stored in PTW at 4°C for several days up to two weeks, which will reduce the background

## Mounting

1. Separate embryos from yolk in PTW
2. Transfer embryos to 87% glycerol
3. Leave in 87% glycerol at least overnight for complete equilibration
4. Mount in 87% glycerol in viewing chamber and take pictures

Comments: Glue one or two small coverslips respectively on a microscope slide (with super glue) to prepare a viewing chamber.

Additional hints

## Preabsorption of antibodies

Embryos need not to be dechorionated

1. Fix embryos in 4% PFA/PTW 4 hrs at room temperature or at 4°C overnight
2. Wash 4 x 5 min in PTW
3. Store embryos in 100% MeOH at -20°C
4. Take 1 ml embryos and rehydrate by washing 3 x 5 min in PTW
5. Transfer embryos to 2 ml Eppendorf tube and homogenize in PTW with a pestle
6. Adjust volume to 1 ml PTW
7. Add 10 µl antibody (final: 1:100) and incubate by shaking at least overnight or store for several days at 4°C
8. Spin down embryonic debris and sterile filter supernatant (cellulose acetate, 0.2 µm)
9. Resuspend embryonic debris in PTW, spin and sterile filter again.
10. Combine filtered antibody solutions and fill up with PTW to desired dilution.
11. Store pre absorbed antibody at 4°C, it will be stable for 2-3 month

Comments: Preabsorbed antibodies can be reused twice

# 7. TANSPLANTATION TECHNIQUES

## 7.1 (General thoughts) Single cell transplantation in zebrafish

### Overview

(Source: R. Ho)

Successful cell transplantation in zebrafish embryos requires a steady hand, a calm spirit, and a knowledge of embryonic anatomy. In addition, concern for the continued good health of the embryos and addition of penicillin and streptomycin to all media are necessary.

Unless one plans to perform transplantation of cells within an individual embryo, separate groups of donor embryos and host embryos are needed. Ordinarily, one should label one of the groups of embryos (usually the donors) so that after transplantation, the transplanted cells can be distinguished from host cells. We have had great success with the family of highly fluorescent dextran compounds, in particular, rhodaminated-dextran, although fluoresceinated-dextran also works. Donor embryos are labeled with glass micropipettes. A pipette is filled with the dye solution and attached to an apparatus that forces the dye out of the pipette with air pressure. The tip of the pipette is then gently broken off, generally by touching it to a pre-shaped glass rod, and inserted into the yolk cell of a donor embryo immersed in 30% Danieau's in a depression slide. A small amount of dye is then expelled into the yolk before withdrawing the pipette. Dye injected before the third cleavage division will be distributed throughout the blastoderm via the cytoplasmic bridges between cells within the embryo. See Blastomere Lineage Analysis, for more detailed methods.

The technique of transplanting cells has taken us many years to perfect and involves the use of specialized transplantation pipettes formed from glass capillaries. Considerable "hands on" skill is required for production of the transplantation pipette. The capillaries are pulled to fine tips on a Flaming-Brown electrode puller. The tips are either broken off at an angle using a hand-held razor blade or beveled with a commercial beveler and then fire polished with a micro-forge. Frequently, a sharp 'spear-tip' is tooled onto the end of the pipette by briefly touching the pipette to the hot wire of the microforge and drawing out the molten glass into a shape resembling that is shown in the figure. This sharp spear-tip is useful for penetrating the outer epithelium of the embryo. A smooth and symmetrical pipette tip is required for successful transplantation. Ideally, the inner diameter should equal the diameter of the transplanted cells. For this reason, pre-formed transplantation pipettes with various diameters should be prepared.

During cell transplantation, arrange the labeled donor embryos and non-labeled host embryos on a depression slide in 3% methyl cellulose or 1.2% agar dissolved in 10% modified Hank's medium and cover with just enough medium to keep them wet. Fill the transplantation pipette with mineral oil and attach it to an oil-filled Hamilton syringe with polyethylene tubing. Mount the transplantation pipette onto a micro-manipulator and position within the labeled donor embryo. Draw cells into the pipette with suction from the Hamilton syringe. Withdraw the pipette, now containing labeled donor cells, and insert

it into the non-labeled host embryo and expel the transplanted cells. Carefully withdraw the pipette from the host embryo and maintain this embryo with standard procedures.

## Random Thoughts on Sutter Pipette Pullers

from Scott Fraser, edited by Eliza Shah 7/29/94.

A typical pull cycle:

1. The glass is held at a weak "pull".
2. The heater melts the glass.
3. When the melting glass is stretched at rate set by "velocity",
   the heater is turned off,
   the strong pull begins, and
   a puff of air is delivered to cool the glass more quickly.

Parameters Defined:

Heat (range 0-999) The current that passes through the heating ribbon. A ramp test should be run to determine the heat setting needed to melt the glass being used, and settings should be within about 50 of the ramp test result. Warning: different filament will burn out at different heat settings. In general, the box filaments will burn out at settings in the 800's and the trough filaments will burn out at settings in the high 400's.

Velocity (range 0-255) A measure of the fluidity of the glass, used as the threshold for the strong pull. Some pullers measure position instead of velocity, which is less accurate.

Time (range 0-255) The duration of a puff of air delivered to the glass and filament to cool the glass. This cooling permits the puller to form very sharp-tipped pipettes that are not long and whippy. Too little cooling may make tip too long and easy to plug, too much can cause the pipette tip to break with a large opening for the tip.

Pull (range 0-255) The strength of the strong pull, applied by an electromagnet. Rule of Thumb:

Longer needles result from:
   Heating the glass quickly
   Pulling the glass more strongly
   Higher velocity settings
Shorter tapers result from more cooling

Complex programs of nested pulling steps can be created. Many of our programs stretch the glass to a waist (hour glass shaped) by applying heat but no strong pull, then a second step with a strong pull creates the tip

---

## 7.2 Following cells in Fish

(contributed by Don Kane)

### Introduction

What with their genetics, zebrafish have become the Drosophila of the vertebrates. But these little fish have many other advantages. As the developmental biologists of the mid- 20th century knew (1), zebrafish are superb for observational studies of development. Their eggs are fertilized externally, so that it is a simple matter to have the embryos on a compound microscope a few moments after fertilization. There, the transparent embryo reveals the early cell cleavages of the blastula, the cell movements of the gastrula, or, later, the forming internal organs of the larva. In situ hybridization and antibody reactions are done as whole mounts, for sectioning can be done later, if at all. And if lineage tracer is injected into cells, as we describe in this chapter, the cells can be recorded for days and sometimes weeks afterwards.

The technique of moving about pieces of tissue from embryo to embryo has been an essential part of developmental biology for nearly a century. By placing developing cells into ectopic positions, we test the potential of the transplanted cells to develop into fates appropriate for the new position. These techniques, described in this chapter, are essential for connecting the developmental biology of the zebrafish to that of more traditional experimental vertebrates, such as Xenopus and chick, and are also necessary for the analysis of gene action in the zebrafish mutants.

In this chapter we first describe some of the general methods for injecting lineage tracers in to the embryo. These techniques can be used for creating labeled embryos (for later experiments) or for determining cell fate. Next we describe methods for moving cells from one embryo to another, methods that are used to test cell fate or cell autonomy. Lastly, we describe methods for the analysis of these experiments.

Many of the methods we describe are imagined as difficult stuff, performed by those possessing calm temperaments and quiet hands. In fact, we have introduced some of these methods into undergraduate laboratory sessions, where students, after dechorionating embryos with watchmaker forceps, label cells with hand held pipettes controlled by breath pressure, and later move cells about with small pieces of cactus spines and broken glass. Most who attempt these techniques a few times will have them working. In fact, the ultimate challenge is not performing the manipulations themselves, but analyzing and interpreting the huge amount of data often produced from seemingly small and simple experiments.

---

## 7.3 Cell labeling techniques

Fate maps define what cells at some early stage later become. These maps are a starting point for the description of the normal development of the embryo. The labeling techniques described in this

subsection are used to construct these maps, and are the methods used for the now classic Kimmel Warga and Schilling fate map of the zebrafish blastula (2). Still, much fate mapping remains in progress, either of finer regions or later stages (3-7). Much of this new work is motivated by the expression patterns of new cloned genes in zebrafish, where we wonder where fate lie in these expression patterns; other work is motivated by mutants that change expression patterns, where we wonder if fates are changed as well. Thus, in both cases, to properly answer the question, we must label cells and ask what they become.

Fluorescent and biotinylated dextran conjugates are the molecules of choice for labeling blastomeres of zebrafish embryos (Table 1). Having a high molecular weight, good water solubility and low toxicity, dextrans are hydrophilic polysaccharides that are stable in the intracellular space. Because dextran conjugates are membrane impermeable, they can be kept in clonal progeny of labeled blastomeres for many days, and, for some early differentiating cells, several weeks.

With a bright red fluorescence, the photostable tetramethylrhodamine dextran is the preferred dye for repeated observation with epi-illumination. While the fluorescein dextrans are often used as a second color, the photostability of the fluorescein conjugates is not as high as that of tetramethyrhodamine dextran and prolonged exposure causes substantial photodamage to the cells, making the dye less desirable for repeated observations by fluorescent microscopy. A rhodamine derivative, RhodoB, is now available that fluoresces with the fluorescein filter set, and this new dye may be a partial replacement for fluorescein. Another alternative color is Texas Red, which can be seen on the rhodamine filter set. Cascade Blue, which uses the UV DAPI/Hoechst filter set, seems an obvious third color; however, its use as a vital dye is somewhat limited because of its UV excitation wave length, which is cytotoxic, and its blue emission, which is invisible to many intensified cameras. Note that most of these dyes come in lysine-fixable versions, allowing covalent linkage to surrounding tissue during fixation.

Due to their low diffusion, newer fluorescent and biotinylated dextrans of super high molecular weight (500,000 and 2,000,000 MW) are particularly useful for fate-mapping blastomeres of the early stage embryo. The lower molecular weight dextrans, under 50 MW, leak from blastomeres during early stages, when the cells are cytoplasmically bridged to the yolk, and then leak from the yolk and label other blastomeres. Another advantage of the large MW dyes is that they are excluded from the nuclei, allowing counterstaining of nuclei to visualize nuclear labels. However, clogging needles and low solubility limit somewhat the convenience of the large molecular weight dyes.

For particularly difficult injection locations, the so called DMNB-caged fluorescein (Molecular Probes, Eugene, Oregon) dextrans can be used to label portions of the embryo. These dyes are injected at early developmental stages, and then later the target cells, which may be small or buried in tissue, are photo-activated with UV, uncaging the fluorescent molecule. Some of these dyes are fixable, e.g., the uncaged fluorescein can be late visualized with the normal antibody against fluorescein.

## Table 1. Common Vital Dyes and Terminolgy.

| | |
|---|---|
| Rhodomine (tetramethyl-rhodomine) | Vital fluorescent dye; Red color when excited with green light; Good for light intensifiers with sensetivities biased toward infrared; excellent resistance to photobleaching and phototoxicity. |
| RhodoB | Vital fluorescent dye; green color when excited with blue light; good resistance to photobleaching and phototoxicity. |
| Texas Red | Vital fluorescent dye; Red color when excited with green light; Good for light intensifiers with sensetivities biased toward infrared; excellent resistance to photobleaching and phototoxicity. |
| Cascade Blue | Vital fluorescent dye; Blue color when excited with UV light; Poor for light intensifiers with sensetivities biased toward infrared; excellent resistance to photobleaching and but UV is toxic over intervals used for time lapse. |
| Fluorescein (fluorescein isothiocynide) | Vital fluorescent dye; Green color when excited with blue light; Good for light intensifiers with sensetivities biased toward infrared; poor resistance to photobleaching; moderate phototoxicity |
| photo activatable dyes.xxx | Vital fluorescent dye; little initial fluorescence; after 'activation' with UV light, the dye gives a green color when excited with blue light. |
| DiO, DiI | Lipohilic dyes for marking cell membranes. |
| Biotin | Added to lineage cocktail for later fixation, amplification with avidin-biotin and visuation with horseradish peroxidase color reaction. |
| 10 000 MW | Normal weight for lineage analysis |
| 70 000 MW | High weight for lineage analysis of marginal blastomeres at 16 to 64 cell stage. |
| 2000 000 MW | Ultra high weight for lineage analysis of 2 to 16 cell stages. |
| Dextran | Sugars covalently bound to tracers to increase molecular weight. |
| Anionic, Cationic | Charge on dextrans to aid in electrophrosing tracers into cells. Neutal dyes are fine for simple pressure injection |

## Labeling whole embryos with lineage tracer dyes

For transplantation techniques, cells from donor embryos must be marked to distinguish them from host cells. In classic work, this labeling was achieved by using donors possessing different genetic characteristics, such as different cytological characteristics (8) or lack of pigment color (9). These days, whole embryos are labeled by injecting fluorescent and biotinylated dextrans into the yolk prior to the 16 cell stage (Figure 1). When the blastomeres of the early embryo are cytoplasmically continuous with the yolk, low molecular weight tracer dyes mix with cytoplasm amongst the yolk platelets and rapidly move into blastomeres carried by the early cytoplasmic streaming of the embryo. Clutches containing mutant embryos can be sub-divided into a number of groups of embryos, and each group labeled with a different color, leaving one group unlabeled for later use as hosts. Embryos, later be used for transplantation, must be dechorionated, either manually with watchmaker forceps or enzymatically with 0.5 mg/ml of ProteinaseK.

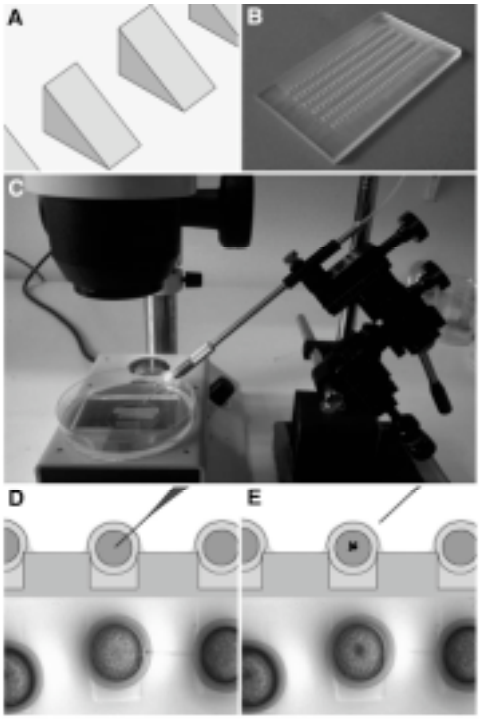



Fig. 1: Single cell injection

Protocols 1 and 2 are appropriate for large scale injections, where hundreds of embryos can be labeled in a single sitting. With little modification, this method is also appropriate for injection of DNA and RNA constructs, or for the injection of pharmaceutical agents that that do not readily enter the embryo, such as ethanol, alpha amanitin or cytochalasin B. Alternate steps are outlined for small scale injections, where tens of embryos are injected, for which we prefer to manually dechorionate the embryos and then inject them in an agar bottomed Petri dish.

With the appropriate lineage tracers, this method can be extended to a crude sort of lineage technique. If a single blastomere is injected with dyes that have molecular weights in the 70 000 kD size range, or in the case of the 2 to 4 cell stage, the 2000 000 kD size range, the dye only very slowly diffuses into the adjacent cells, effectively labeling the progeny of the targeted cell. For general lineage work, these clones are too large to analyze because of the large region of fluorescent label which occludes underlying cells, and because these early clones contribute to the majority of the tissues of the embryo. However, the method is very useful as a teaching aid (10), and in certain experiments, it is useful in the marking of co-injected DNA or RNA constructs (11).

## Protocol 1. Agarose plates for holding embryos

### Equipment and reagents

- 60 or 100 mm plastic Petri dishes
- 1.5% agarose solution (in E2 medium). (Check note on agar media in Table 2)
- Plastic mold or glass 1 mm to 1.5 mm capillary pipettes or glass depression slides.

### Method

1. Pour liquefied 1.5% agarose into plastic Petri dish to a depth of about 5 mm.

2a. Float a plastic mold into the agarose taking care to avoid trapping air bubbles below the mold.

2b Alternate. Float several capillaries (1 mm to 1.5 mm diameter) in the liquid agar.

2c Alternate. Tip a thick glass microscope slide (such as a depression slide) from the bottom of the dish to the edge, to form a trough in the liquid agar.

3. When the agarose is completely solidified, gently lift the mold or capillaries or slide away from the agarose using coarse forceps or a sturdy needle.

## Protocol 2. Early Cleavage Injections

### Equipment and reagents

- Dissecting microscope, equipped with transmitted light.
- Micropipettes. Make micro pipettes from 1.0 or 1.2 mm thin walled glass capillary glass with filaments (e.g. TW120F-4 from World Precision Instruments) The capillary is pulled by an electrode puller (e.g. Flaming Brown) to produce a fine needle on the tip. This needle should be thick enough to penetrate the chorion without breaking, but thin and long enough (about 10 µm thickness and 1 mm length) so the embryos are not dragged when the needle is withdrawn from the chorion. In the alternate method, (not penetrating the chorion) the needle can be thinner.
- Micromanipulator. One movement should be set so that the micro pipette moves parallel to the injection needle.
- Micro injector. Most pressure injectors will work; we prefer models that are compact and manually controlled. A simple and adequate setup is that by ASI with a pressure gauge, a switch (for pressure on/off), and mechanical pressure regulator. The unit should be able to attain 50 PSI.
- Agarose plates (Protocol 1)
- Fine forceps

### Method

1a. Place 1-16 cell stage embryos with chorions in the holes or slots in the agarose plate. If holes, each hole can hold 1 embryo. As much as possible, remove extra medium on the agarose plate with a pipette. Embryos will be held firmly in the holes by the surface tension of the medium.

1b. Alternate. Dechorionate the embryos and place in an agar mold, or on a plain agar plate. In this case do not remove extra media.

2. Fill the micropipette with the dye solution. The capillary will draw the solution to the tip of the pipette. Special thin gel loader tips (e.g. Eppendorf Micro loader) are sometimes useful for this step. This step can be done the evening before the injections and the needles stored in a humid chamber; extra time helps the tips fill better and allows air bubbles at the tip of the pipette to escape.

4. Set the micropipette in the needle holder connected to the pressure injector.

5. Break the tip of the micropipette with forceps or by touching lightly against a piece of cover slip.

6. Place the tip in a droplet of mineral oil, and work the injector to make a small droplet of dye solution. Adjust output pressure and injection time, so that the size of the droplet is between 50-60 µm (roughly 1/10 the diameter of the yolk cell).

7. Place the agarose plate with embryos on the stage. Move the micropipette tip to penetrate the chorion and place the tip at the center of the yolk by using the micromanipulator and/or by movement of the agarose plate.

8. Inject the dye solution by pressure, and withdraw the micropipette from the embryo.

9. Repeat steps 6-8 for each embryo.

10. Add E2 medium on the agarose plate. Collect and transfer the embryos from the agarose plate to Petri dishes containing E2 medium.

11. Incubate the injected embryos away from direct light.

---

## Labeling individual blastomeres after 256 cell stage

Cells labeled before the late blastula or gastrula stage tend to give rise to many unrelated tissue types. This is due in part to the short cell cycle times in the early blastula that produce some 20 to 50 cells and

in part to the spreading into many different positions on the fate map driven by the radial intercalation movements of early epiboly. Labeling shortly after the 1,000 cell stage does not eliminate these problems--epiboly is yet to begin--but it helps to manage the problem to a smaller number of cells that tend to remain together. The methods described here have been used to create the blastula fate map. For later fate maps, this method has been extended into the gastrula and segmentation periods (7,12). These later maps have less variability because the movements of local regions of the embryos become more predictable, and cell mixing is less of a problem.

To label cells after the 1,000 cell stage, we resort to the technical methods of the cell physiologist, using electronics to enter the cell and even to inject dye. The general method, outlined in Protocol 3, is to place the tip of the pipette on the cell membrane of the cell to be injected, depolarize the cell membrane which creates a very small hole through which the pipette enters, and then use a combination of pressure and current to push dye into the cell.

Cells labeled in this manner often give rise to two related clones, one bright and one dim (See clone in Figure 7). The brightly labeled clone is descended from the injected cell; the weakly labled clone is descended from the brightly labeled cell's sister, which was labeled by leakage of dye across the midbody remaining from the mitotic division preceding the injection. Because cell divisions at the early blastula cell stages tend to give rise to widely separated siblings, in this case the founder cells of each clone, these sub clones can sometimes be mapped as independent clones. To avoid such dual clones, inject cells that have rounded up for mitosis; these cells have usually lost their midbody remnant.

## Protocol 3. Late cleavage injections.

### Equipment and reagents

- Microscope: Upright design equipped with DIC optics, epifluorescence, 10x and 20x dry objective lenses and 40x or 63x water immersion objective lenses.
- Micromanipulator: With fixed stage scopes, any of the heavy mechanical micromanipulators (such as those manufactured by Leitz) are acceptable. If the scope has a movable stage then a light micromanipulator (such as those manufactured by Narishige) is necessary. The micromanipulators should set with the z-direction square to the horizontal (aligned with the movement direction of the objective) so that movement along this axis causes no apparent x and y movement when watching the pipette through the microscope. In the case of the stage mounted manipulators, it is very useful to have the z-direction controlled hydraulically such as in the Narishige MO155. In all cases, besides x, y and z, a fourth hydraulically controlled movement aligned along the shaft of the pipette is very useful, allowing the simple retraction of the injection pipette out of the cell after injections.
- Micro injector as in Protocol 2.
- Electronics. The electronics set up is typical for cell physiology; expertise and advice on these techniques is often found in local Neuroscience departments. The central apparatus consists of an intercellular amplifier that can detect and control the voltage and current at the tip of the injection pipette. An essential accessory is an electronics oscilloscope to visualize the output of the intercellular amplifier. The equipment should be able to measure current at levels of 2 mA. Also useful, are the so called 'cap comp' electronics, and a stimulator to control the amplifier to deliver a 10 millisecond voltage spike of about 10 volts, both used for clearing clogged pipettes or entering recalcitrant cells. Completing the circuit is, on the pipette side, the pipette itself (back filled with 1 M KCl) in a pipette holder with an silver bridge electrode holder (such as a World Precision Instruments MEH900S), and, on the bath side, a silver chloride agar bridge, made by inserting a silver wire into a 10 cm long plastic capillary filled with 1.5 % agar in 1 M KCl.

- Glass pipettes. Preparation of proper glass pipettes for injection is crucial for this procedure. Again, a Neuroscience department often has the personnel that can help the inexperienced. Use1.2 mm thin walled borosilicate capillary glass filaments (e.g. TW120F-4 from World Precision Instruments). Using a electrode puller, such as the Flaming Brown P80/PC electrode puller, this glass is pulled with a steep shank to produce a moderately stiff needle with a little whip. For late blastula labels (at the 1,000 through 4,000 cell stages), the resistance of the pulled needle should be over 300 meg-ohm. Immediately before use, the tips of these needles are broken to a resistance of about 120 meg ohms on a glass ball. For gastrula labels, pull thick walled borosilicate capillary glass that has a hole that results in a resistance of about 100 to 300 meg ohm, trying to make a very steep shank, producing a stiff needle with very little whip. Gastrula needles will not be broken before use. Using the capillary of the pipette, the pipettes can be back filled several hours prior to (or the night before) the experiment, and stored at 100% humidity. In general, the fine glass tips of the capillaries dull on storage; thus, the glass should be pulled within 48 h of experiment.

### Method

1. Dechorionate embryos.
2. Break pipette against glass ball (if injecting at the blastula stage).
3. Place embryos in a depression slide in E2 medium.
4. Focus microscope on the nucleus of the cell to be labeled.
5. Bring focus on the lens up about 1 to 2 mm.
6. Position pipette in light path and lower towards embryo, chasing the z-position of the needle with the plane of focus of the microscope.
7. Place the pipette above the cell and then lower the needle against the cell, dimpling it.
8. Depolarize cell membrane using a voltage pulse.
9. Verify that the pipette is in the cell (based on a change in voltage).
10. Inject using pressure or electrophoresis.
11. Back the needle out of the cell using axial drive.

---

## Labeling groups of cells by photoactivation

One method that has been gaining popularity in recent times is the marking of cells by photoactivation of a dye that was initially injected into the entire embryo (13,14). This method has the advantage that cells that are difficult to access with an injection needle can be labeled, and embryos are not traumatized during the sensitive gastrula stages by an injection needle. The labeling of the initial clone is best accomplished with a laser focused through the objective lenses of a compound microscope; an adequate replacement is to activate using a small pinhole in the aperture of the UV epi-illumination, as we outline in Protocol 4.

Typically, the labeled cells are not true clones, in that initially more than one cell is activated (or 'labeled'). Also, after repeated illumination, the activated cells and the unactivated cells immediately surrounding them seem to slowly assume the same level of fluorescence. Nevertheless, the method is ideal for intermediate fate maps, where lineage restrictions are not being tested and where vector movements of tissue layers are being analyzed.

One useful variation of this method is to co-inject a rhodamine labeled dye with the photoactivatable dye during late cleavage, and, at a later time point, activate the photoactivatable dye in a portion of the

clone. This method produces a small sub-clone of cells which is framed by the movements of parent clone. This variation gives rise to a more manageable number of progeny, akin to a late blastula lineage label.

---

## Protocol 4. FITC photoactivation

### Equipment and reagents

- Microscope. Compound microscope equipped with epifluorescent illumination, 20x dry (0.5 NA), 40xW (0.7 - 0.9 NA) and 63xW (0.8 - 1.0 NA). DIC optics and epifluorescence for rhodamine, FITC, and DAPI/Hoechst. There must be an aperture at the virtual image of the epifluorescence path that reduces the UV beam to a narrow beam. If there is no aperture, introduce a slider at the appropriate position containing foil with a pin hole aperture. It is also helpful to have an appropriate combination of neutral density filters and an aperture at the virtual focus of the epifluorescence path to control UV intensity. An alternative method for photoactivation, not discussed here, is to activate the cells using a focused beam from a laser.

### Method

1. Inject embryos during early cleavage with caged dye.
2. Incubate embryos to desired stage. Protect from light.
3. Using the highest practical power/N.A. objective, focus in the embryo the precise location to be labeled. Use the FITC filter with the aperture closed to a pin hole to establish the area to be labeled.
4. Turn off the epifluorescence and slide the DAPI/Hoechst filter set in.
5. Briefly illuminate the specimen, observing the change in color visually.

---

## Labeling groups of cells by lipophilic membrane dyes

An alternative cell labeling technique is to label the cell membrane of cells. This is typically done with the fluorescent dyes DiI and DiO, which can be visualized with the rhodamine and fluoresceine filter sets, respectively (15). These dyes, if injected or 'stabbed' with a tungsten needle into the embryo, will incorporate into the bi-lipid cell membrane of nearby cells. As for photoactivation, the labeled cells are not clones, in that more than one cell is typically labeled. However, this is a particularly nice method to label cells or axons that migrate from the label site, and the method has been used successfully to label migrating neural crest cells and the migrating growth cones and axons of the motoneurons of the spinal cord.

---

# 7.4 Transplantation techniques

A central question in many developmental biology problems is knowing when and where cells begin to irreversible enter a differentiated state. To test such ideas, we describe here methods for moving cells

from one place in an embryo to another place in another embryo, testing the state of commitment of cells (16).

The transplantation technique is also essential in the determination of the autonomous and nonautonomous effects of gene activity (17). Here the technique is used to determine which cells are expressing the gene of interest and what effects these cells have on other cells in the embryo that are not expressing the gene. Hence, the transplantation is not from location to location but from one genotype, to another.

In both of the above cases, we are looking for differences in the fate of the transplanted cells. For most normal work, the use of double transplantations, where the behavior and fate of one group of 'experimental' cells is compared with a 'reference' control transplant, reveals slight differences in movement or fate between the groups. When looking for cell autonomous effects of mutations, the double transplant method also has the advantage that the number of hosts containing mutant cells nearly doubles, which doubles the efficiency of documentation on subsequent recording sessions.

Lastly, transplantations can, with care, be used for fate mapping. This method had been the method of choice for classic work in amphibian and chick fate maps. At late stages, moving small labeled cells is easier than injecting them. While these late transplantations must be homotopic, and control experiments with actual injections would be necessary, transplantations could be used to form the bulk data set.

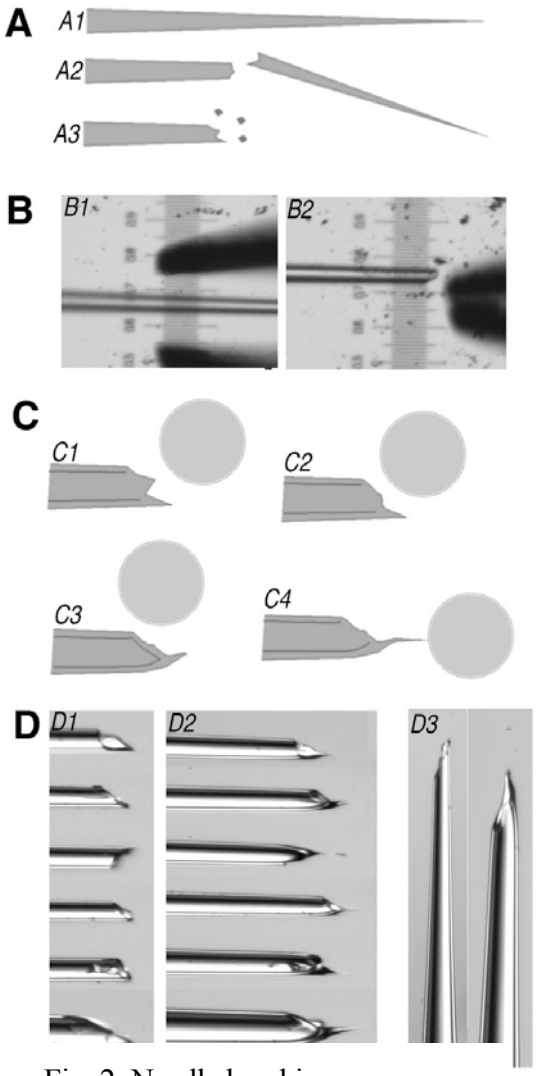


Fig. 2: Needle breaking

## Precision transplantation of small groups of cells

Transplantation of single cells is a superb test of cellular determination. Large groups of cells and large pieces of tissues may contain many of the intracellular components that may have initially conferred fate onto the tissue. A single cell has a far better chance of losing such intracellular baggage. Actually, in the zebrafish, even somewhat larger transplantations, in the range of 10 to 25 cells, are also an acceptable test, as the introduced group of donor cells normally spreads and mixes into the surrounding host tissue, and the intracellular material is certainly diluted. Even in the case of single cell transplantations, some extracellular material is also drawn into the pipette. To advantage or not, this material may be important for the immediate survival of the blastomeres: single blastomeres often begin to lyse when the transplantations are done in a leisurely fashion, perhaps because of diffusion of the extracellular melee.

Protocol 5 and Figure 2 outline the construction of the transplantation pipette. Basically, the pipette is crudely broken to a spear tip of the correct diameter, and then the end is smoothed and sharpened on a microforge. The 'perfect size' for the pipette is such that the blastomeres just fill the bore. Our simplistic chipping method for fashioning the spear can be replaced by grinding on a needle beveler, such as supplied by Narishige. The fashioning of a spear tip with a sharp whisker allows easy entry through the enveloping layer of the blastoderm. Without the whisker, the pipette tends to push the embryo out of the holding preparation. However, if an agar mold is used to hold the embryo, then a large bore broken and chipped pipette can be used without microforging.

Small groups of cells can be moved between labeled donors and unlabeled hosts (Protocol 6 and Figure 3). The embryos are immobilized in methyl cellulose during the transplantation operations. Typically, for the most accurate placement of cells, these operations are performed under DIC illumination using a 10x to 20x objective. With a properly fashioned transplantation pipette, the pipette enters the embryo using a fine glass whisker to penetrate the enveloping layer, and then, using suction, deep blastomeres are gently drawn into the pipette. Then, after leaving the donor and entering the host embryo, the end of the pipette can be positioned in the appropriate place of the embryo and the cells expelled.

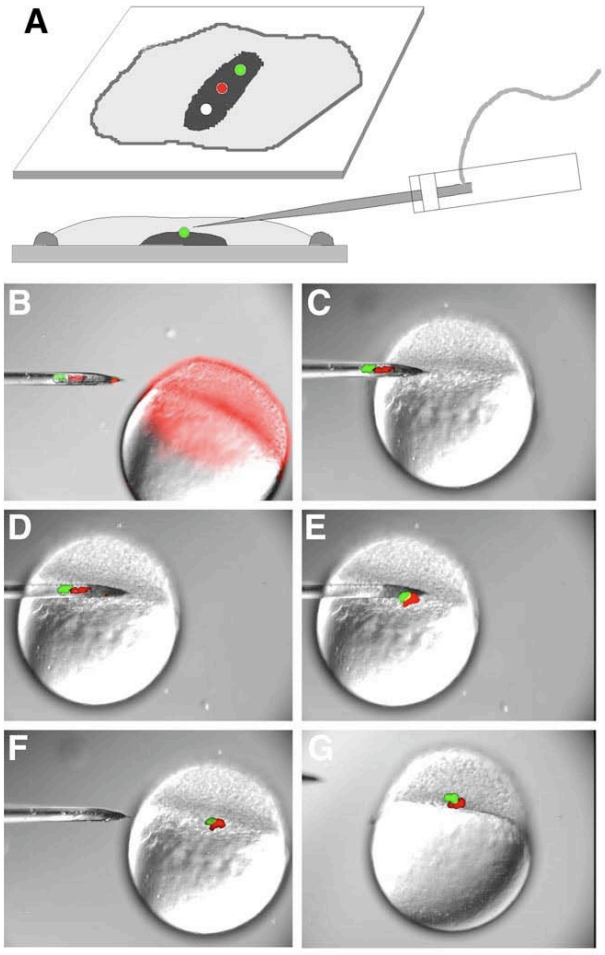



Fig. 3: Transplantation method

The transplantations are easiest to perform between 30% to 50% epiboly, a period when the blastomeres are quite loose, and, also at this time, the geometry of the embryo allows easy placement of the blastomeres. These conditions are very important when targeting cells to the mesodermal and endodermal tissues, because cells must be placed very close to the blastoderm margin. What with the ideal period for doing the transplantations so short, it is most efficient to use two stations, one at a dissecting scope for mounting the embryos and one at compound scope for performing the transplantations.

With care, experiments can be done before and after the optimal times. Before 30% epiboly, the blastomeres are very fragile and the cell lethality is high (the cells have the handling characteristics of water balloons). Also, because of the rapid cell cycle in the early blastula, the blastomeres are quickly decreasing in size throughout the transplantation period, necessitating frequent changes to smaller transfer pipettes.

In later transplantations, i.e. after 50% epiboly, the blastomeres begin to become sticky and difficult to draw into the transfer pipette. Also, as the cell layers in the gastrula become thinner than the outer diameter of the transfer pipette, it is easy to injure the yolk cell. However, after this 'difficult' period, transplantations become easier as the embryo acquires axial thickness in the mid segmentation stages, and transplantation of neural crest progenitors is quite possible.

While more time consuming than that for large scale transplantations, this method is preferred for initial training in the cell transplantation technique. Seeing the tip of the pipette, the embryo, and the cells and their nuclei helps to quickly correct common mistakes in manufacture of the transfer pipette and treatment of the embryos.

---

## Protocol 5. Construction of transfer pipettes.

### Equipment and reagents

- Dissection microscope
- Pipette Puller
- Microforge
- Thin walled glass pipettes
- Razor blades or fine forceps.
- Stage micrometer or calibrated eyepiece ocular grid.

### Method

1. Pull pipette from thin walled glass tubing (OD 1.0 to 1.5 mm) with no capillary. The shape of the pipette should have a long taper, narrowing from 10 µm to 100 µm over about 5 mm.
2. Using a razor blade, break the glass to the appropriate diameter (OD of about 40 µm for late blastula work).
3. Working under a dissecting scope, chip back glass until the tip is spear shaped. At this point the glass can be used for crude transplantation experiments.
4. Using a microforge, melt the glass at the edges of the opening.
5. Using a microforge, fashion a fine glass whisker on the tip of the spear by lightly touching the pipette to the microforge filament and pulling away.

---

---

## Protocol 6. Single cell transplantation

### Equipment and reagents

- Room. The room that the microscope is placed in should be of uniform temperature and free of drafts. Temperature shifts will cause fluctuations in the movement of the oil in the micro injector. In a drafty room, it is sometimes helpful to construct a box around the sides and back of the microscope.
- Microscope. Upright compound microscope equipped with DIC optics and a 10x lens. A left handed mechanical stage allows the micromanipulator to be placed on the right side. Note that the mechanical stage should be smooth and have no backlash. Epifluorescent illumination is recommended.
- Micromanipulator. If not using a fixed stage scope, the micromanipulator must move with the stage. A fixed stage microscope and a table mounted micromanipulator are recommended but expensive. A

light manipulator, such as the Narishige MN151, attached to the stage mount of a normal compound scope is quite acceptableand inexpensive. Set the angle of the micro manipulator to about 15 degrees to perpendicular.

- Micro injector. The cells at the tip of the pipette are controlled hydraulically using a hydraulic micro injector, such as the Narishige IM-5B or the Eppendorf Cell Tram. An inexpensive injector can be made with a Hamilton 1702TRLT Threaded Syringe attached to about 60 cm of stiff tubing ending in a World Precision Instruments MPH4 or MPH3 pipette holder, taking care to epoxy the tubing to the syringe and pipette holders. Carefully inspect the entire apparatus and remove any small air bubbles.
- 3% methyl cellulose
- Cover slips, microscope slides, forceps
- Vasoline
- E2 or Danieau's. All media must contain antibiotics.

### Method

1. Dechorionate embryos.
2. Inject embryos with lineage tracer before the 16 cell stage.
3. Prepare 22x22 mm cover slips with Vasoline rings.
4. Wet adjust transfer pipette in droplet on cover slip. Set up so that the pipette can be backed out of the droplet using the x-axis drive allowing enough room to move the cover slip in and out. Adjust the oil meniscus to the tip of the pipette.
5. For each set of embryos:
   - Place a 4 x 10 mm swath of 3% methyl cellulose in the center of the cover slip.
   - Place a droplet of medium (E2 or Danieau's) over the methyl cellulose.
   - Position embryo in methyl cellulose, arranging donor and host embryos in a line perpendicular to the transfer pipette.
6. Advance the transfer pipette into the droplet, positioning the tip of the needle over the donor embryo.
7. Lower the pipette to dimple the surface of the embryo and advance the needle sliding it into the embryo, taking care to not nick the yolk cell.
8. Back oil meniscus into pipette, pulling cells into pipette.
9. Slide pipette out of embryo.
10. Repeat steps 7 - 9 for additional donors as desired.
11. Repeat steps 7 to 9 for host, only expelling cells in step 8 by advancing the oil meniscus.
12. Transfer coverslips to 50 mm Petri dishes filled with E2 medium. The coverslips can be floated on the top of the medium until the embryos have completed epiboly and later transferred to the bottom of the Petri dish and the cover slip removed.

---

## Large scale transplantations of early blastomeres

When the difficulty of placing donor cells into a particular host tissue increases, the desire for greater numbers of experiments increases. The fine scale transplantation technique normally yields about 15 to 30 hosts, and using two donors per host only doubles this number. By using dissecting scopes, simple micromanipulators, and agar plates, hundreds of experiments can be done in a single sitting. Furthermore, many workers tend to favor the convenience of using their own dissecting scopes for transplantation

experiments rather than competing for the laboratory compound scope. Thus, the large scale method outlined in Protocol 7 has become quite popular.

Like in the fine transplantation technique, cells are moved with a hydraulically controlled pipette between labeled donors and unlabeled hosts. However, during the transplantation operations the embryos are immobilized in various types of crafted agarose surfaces and the operations are typically performed under 50x on a dissecting scope. Because the field of view is larger and the depth of focus is greater, the pipette can be quickly moved from one embryo to the next.

These transplantations are easiest to perform in the late blastula, a period when the large diameter transfer pipette can be kept away from the large yolk cell. Although at this time the geometry of the embryo does not allow easy placement of the blastomeres, the scatter of the blastomeres during epiboly tends to distribute the donor cells to many tissues of the hosts.

The misplacement and death of embryos can be rather high in this experiment. 'Jumping' of embryos from one hole to another can be controlled with a layer of methyl cellulose. Misplacement errors can be minimized using an alternative experimental design where only wild type embryos are labeled and cells are transferred into host embryos produced from mutant clutches. Thus, all unlabeled embryos in a Petri dish are hosts. For the survival of the embryos, the use of antibiotics at all stages after the operation is essential. To prevent cross infection from dying embryos, embryos should be segregated into individual petri dishes or wells by the early segmentation stages.

---

## Protocol 7. Large scale transplantation.

### Equipment and reagents

- Room, Micropipettes, Micro injector. As in Protocol 6.
- Dissecting microscope, equipped with transmitted light, and powers from 20x to 50x.
- Micromanipulator, such as the Narishige MN151. Set the angle of the micro manipulator to about 45 degrees to perpendicular.
- 3% methyl cellulose
- Agarose plates (Protocol 1)
- Watch maker forceps

### Method

1. Collect, dechorionate, and label embryos as in Protocol 6.
2. Prepare the agarose plates by placing a bed of methyl cellulose in the troughs or holes, afterwards scraping off the excess from the agar surface. Overlay the plate with 2 -5 mm of medium.
3. Transfer donor and host embryos in troughs of agarose plate, so donor and host embryos are placed side by side.
4. Place the agarose plate under the dissecting microscope and move the agarose plate and the micropipette so that the tip of the pipette is touching the one labeled donor embryo.

5. Insert the micropipette into the one labeled donor embryo, and draw cells into the pipette. Withdraw the pipette from the donor embryo.
6. If using a second donor embryo, repeat step 6 with the next embryo.
7. Place the tip of the micropipette near the surface of a host embryo.
8. Insert the micropipette and transplant the donor cells into the host embryo.
9. Withdraw the pipette from the host embryo.
10. If possible, allow the embryos to remain undisturbed until epiboly is completed. Then transfer the embryos to small agar dishes or microtiter plates, one embryo to a dish or well.

---

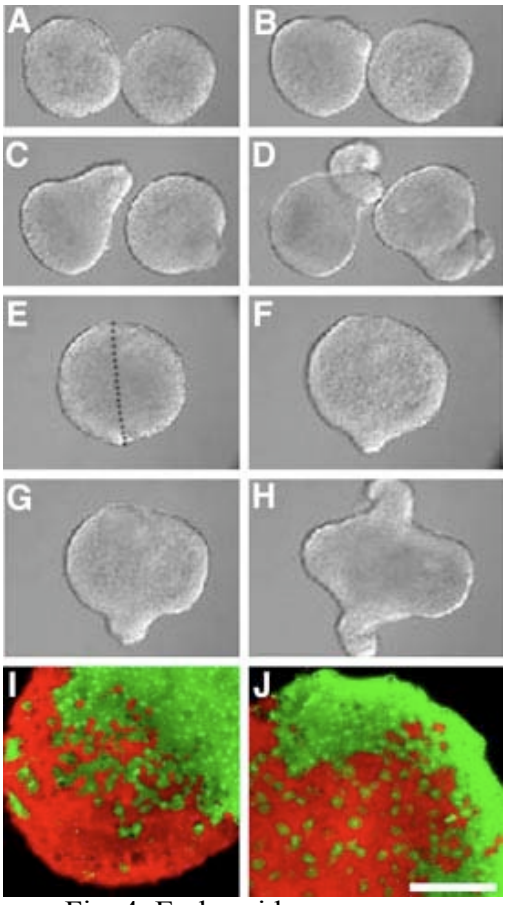


Fig. 4: Embryoids

## Transplantation of pieces of embryos

In certain experiments it is desirable to transfer or remove a portion of the embryo including the intracellular environment. In this case, the cells must stay together, forming a small community. One method to accomplish this is to replace the deep cell domain with a large group of transplanted deep cells, creating a chimera in the embryo in which a portion is mutant and a portion is host. This can be accomplished by aspirating half to three quarters of the cells out of the host embryos and then transplanting half to three quarters of the cells of a donor blastoderm into the host embryo, using one donor for each experiment. Large groups of cells moved in this manner mix only at the border between the donor and the host cells. However, in such an experiment, intracellular factors are dispersed.

An alternative method is to transplant pieces of embryos. Using cactus or tungsten needles, the blastoderm can be dissected off a 128-cell embryo at 2 h post fertilization and placed on the yolk cell of another embryo from which the blastoderm had been previously removed. Using a small piece of agar, or a hole in an agar surface, the donor blastoderm can then be held in place until it has healed in. In these experiments there is a ring of cells along the margin of the blastoderm that are derived from the yolk cell before the formation of the yolk syncytial layer, but the majority of the blastoderm is from the donor.

When zebrafish blastoderms are removed from the yolk cell after the 32-cell stage and placed in Danieau's medium (18), the blastoderms continue to develop, generating stereotypic morphogenetic movements as shown in Fig. 4. Later, although histogenesis is difficult to interpret, similar to experiments in *Fundulus* (19), markers of cell specification aid in the analysis of the embryoids (20). One interesting variation of this procedure is to heal two blastoderms together, ventral face to ventral face, and examine the interaction of cells at the interface. An example of this is shown in Figure 4E-5J.

## 7.5 Procedures for observing labeled cells

Data analysis is a challenging aspect of clonal and cell transplantation experiments. A single labeled cell at the blastula stage can divide into a 24 hour clone containing some 20 to 60 cells. Each of these cells assumes a unique location, and an unique cellular morphology, amongst more than 150 different possible histotypes.

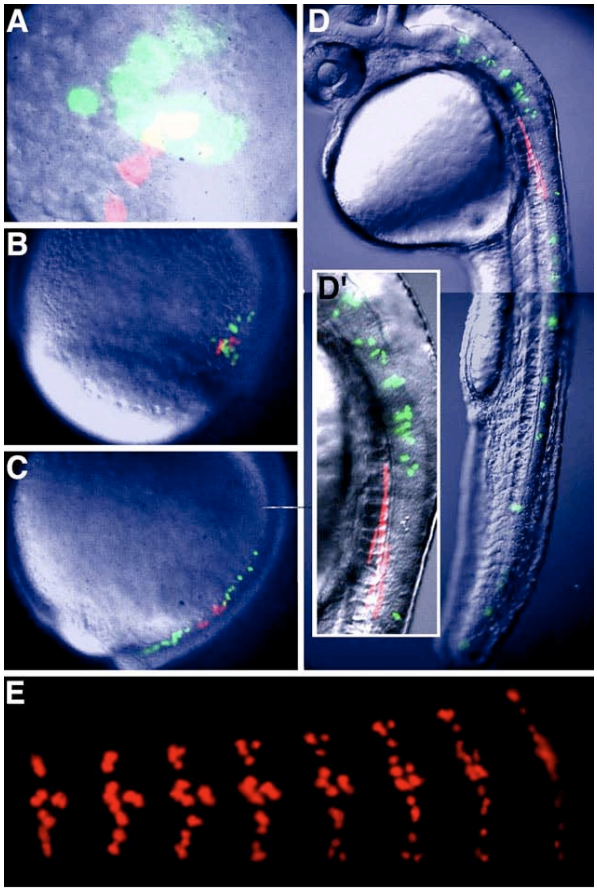



Fig. 5: Cell behavior

### Procedures for observing live material.

For many experiments, intermediate observations of the labeled material are desired. In some cases, the original coordinates for fate mapping must be collected in the late blastula and early gastrula. In other cases, intermediate observations document cell trajectories during gastrulation (Figure 5). And in other cases yet, observations of intermediate morphologies are necessary for the documentation of late differentiating cells as they assume their final morphologies (Figure 6).

For these intermediate observations, we image cells visualized with fluorescence and recorded with low light cameras, using either silicon intensified target cameras or light intensifiers attached to normal cameras. These devices amplify light with quantum efficiency, capturing about 50% of the light photons emitted by the sample to produce a usable image. Other cameras, such as cooled CCD cameras will capture low light images, but only with long scan times or with higher intensity light-lysing the labeled cells in the process. Note that, having been developed by the military to see heat emitting mammals in the infrared spectrum, most of the intensified cameras work best at the red side of visible spectrum.

Protocol 8 outlines many different methods for mounting embryos for observations. Of them, two of the procedures are commonly used during clonal analysis. In the first, we record the starting position of cells relative to the dorsal side of the embryo at about 6 hours development. This requires two views, one from the animal pole and one from a side view. The animal pole view must be square and record the distance between the shield and the labeled cells. The side view must record the number of tiers of cells between the margin of the

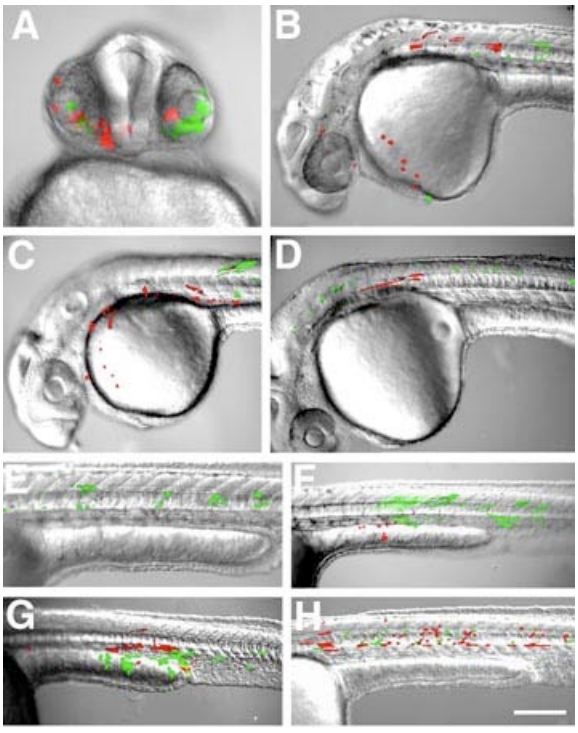



Fig. 6: Histology

blastoderm and the labeled cells. Both these views are typically mounted with methylcellulose and recorded with the 0.30 N.A. / 10x objective of a compound scope. Working with embryos at this stage in methyl cellulose requires practice and patience. For finding the clones and orienting the embryos, we have found the new epilumination dissecting scopes to be very helpful.

The second procedure is the quick scan of 30 hour fish. For this we use bridged coverslips. The best are made with a two pairs of #1.5 22x22 cover slips, one pair glued on each side of a 24x60 coverslip. The glass surface of the coverslip spacers slides better and does not deteriorate, allowing one bridged cover slip to be used for an entire session. The fish are anesthetized by transferring them to a solution containing MS322 for one or two minutes. Then, using a Pasteur pipette, the fish are transferred in between cover slips where they lay nicely on their sides. This side view is very useful for marking the location of labeled material. If there are cells on the 'wrong' side of the embryo, the coverslip can be flipped and looked at from the other side. For the majority of the cells in the embryo, the location of the progeny of the labeled cells will remain unchanged. Hence this is a good starting point for observations which might continue through the larval period.

For microscopy, most brands of microscope work but many lenses do not. The dry 16x or 20x lenses are adequate for scanning the embryo. But for cellular morphology, the 40x or 63x water immersion lenses, with N.A.s of 0.8 to 1.0, are essential. The dry version of the 40x and the glycerol/oil versions of the 63x lenses do not have the working distance to get deep into the preparation. More importantly, perhaps because of the of thickness of the sample, the water lenses seem to process superior optics compared to their counterparts. Note here we are referring to live observations; all lenses work equally well on dead fixed tissue.

---

## Protocol 8 Observing Living Fluorescent Cells

### Equipment and reagents

- Microscope. Compound microscope equipped with epifluorescent illumination, 5x, 10x, 20x 40x dry, and 40xW (0.7 - 0.9 NA) or 63xW (0.8 - 1.0 NA). DIC optics and epifluorescence for rhodamine and FITC. It is also helpful to have an appropriate combination of neutral density filters and an aperture at the virtual focus of the epifluorescence path to control UV intensity.
- Cameras. Any camera that will capture faint UV images. For intermediate recordings, low light intensity and short duration are necessary (especially at the FITC wavelengths), therefore intensified cameras are usually the better choice. However, if the specimens are fixed or about to be fixed, cooled CCD cameras are excellent. Also, if the cells are somewhat over labeled, we have had success with black and white high-resolution cameras from DAGE. The images are typically digitized through a video capture board onto a computer, and saved digitally.
- Dissecting scope.
- 65x25 mm coverslips with 400 □m spacers for 24 h embryos or 600 µm spacers for younger embryos. The best 400 um spacers are two 1.5 coverslips.
- Boiled 0.1 % agarose. Note, this medium appears as a so-called 'semi' solid. However, it is not liquid; not only do the particles of unmelted agar harm embryos, but the agarose will not hold the embryo on cooling. Thus, be sure that the medium has actually been boiled.
- Brass or steel block, for quickly cooling agar preparations to room temperature.

## Method

1a. For oriented views of embryos, mount with methyl cellulose. Lay a 2 mm by 10 mm by 0.5 mm high bed of 3% methyl cellulose on the coverslip in the middle of a Vasoline ring; overlay the methyl cellulose with 0.5 ml medium, using the ring of Vasoline to prevent spreading of the medium. Working under a dissecting scope, partially sink the embryos into the surface of the methyl cellulose, manipulating the embryos using dull forceps or nylon 'hair' loops. This preparation can be observed directly with low power lenses or cover slipped for higher power views.

1b. For quick side views of 24 h to 72 h embryos, anesthetize embryos in a solution of 0.01 mg/ml MS322, and slip in-between coverslips.

1c. For immobilizing embryos for repeated observations (or time lapse recordings), mount the embryos between coverslips in 0.1% agarose. Ring the agarose with a wall of Vasoline to prevent evaporation.

1d. For immobilizing embryos in oriented views, mount in 0.1% agarose in small 0.1 ml droplets. Working under a dissecting scope, as the agarose solidifies, manipulate each embryo using dull forceps or nylon 'hair' loops. If more embryos are to be added to the preparation, add each to a separate droplet, orienting each embryo before adding the next. Ring the preparation with a wall of Vasoline to prevent evaporation. Then over lay the entire preparation with 0.1% agarose and cover with a coverslip. Wait at least 5 min before moving the preparation to allow the agarose to completely solidify.

2. Observations using UV epiflorescence must use low illumination and be short, especially when using the blue FITC filter set. When scanning the embryo, use the 10x objective; low numerical aperture lenses are less damaging to labeled material. At high power, do not use the UV to find cells and do not 'study' the illuminated embryo. Learn to use the intensified camera to observe the embryo and not the eyepieces. (The intensified camera can see dimmer cells at lower levels than human eyes.)

3. Rescue embryos from the preparations by immersing in a 150 mm Petri dish with 10 to 15 ml of medium. Carefully separate the coverslips under the surface of the medium, watching the embryos using a dissecting scope. For preparations made as in 1a, the embryos can be swept from in-between the cover slips with a stream of medium from a Pasteur pipette.

---

## Procedures for preparing fixed material.

In many cases, fixed material is desired, either for a permanent record or because there is inadequate time for immediate analysis. Sometimes particular views require sectioning or cutting away part of the animal. It is possible to visualize the florescently labeled cells in fixed material. However, conventional staining is easier to see in a complex tissue with DIC optics rather than fluorescence. Also, being more sensitive than the florescence, the biotin staining reveals dimly labeled cells.

After following the embryos using florescence, a common preparation has rhodamine/biotin in one group of cells, and fluorescein in another group of cells. Typically at the end of the experiment, the fish are anesthetized with MS322, and fixed with 4% para formaldehyde for 2 to 3 hours at room

temperature or overnight at 4 °C. If the embryos are younger that tailbud stage, the fixation step should be done on an agar surface to prevent damage to the yolk cell.

Our basic protocol for the double staining of fixed material (Protocol 9) can be edited to include many variations. The simplest is to stain only the biotin of one clone; in this case, the cobalt could be eliminated, leaving the brown DAB staining. (This variation is an excellent protocol for use in an advanced undergraduate laboratory.) Sometimes, an antibody reaction is necessary to reveal some useful marker; if the final amplification is planned to be done with avidin/biotin, then the biotin labeled cells will come up in the final staining reaction. Alternatively, the antibody reaction could be done first with cobalt (or one of the many other color reactions available) and biotin be used for the counter staining of the clone. Figure 7 shows several examples of these stains.

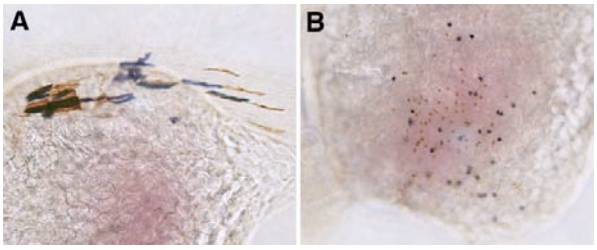



Figure 7: Stained embryos

The mounting procedure is a dehydration into Permont (Protocol 10). As this preparation ages, we have had good experience with the preservation of the DAB and DAB-coca labeled material, as well as many other stains. Note that many dyes fade over several days or weeks if the preparations are stored in solutions of benzyl benzoate and benzyl alcohol.

---

## Protocol 9. Procedure for whole-mount staining of biotinylated and fluorescein dextran labeled cells

### Equipment and reagents

- ice cold acetone
- Elite or Standard ABC kit (Vector Laboratory)
- PBST must be made with TritonX100 instead of Tween20 if doing the CoCl intensification.
- DAB/CoCl presoak solution: add 25 µl DAB stock solution (40 mg/ml in water) and 10 µl CoCl stock solution (60 mg/ml in water) to 2 ml PBST.
- Final concentration of DAB is 0.5 mg/ml and final concentration of CoCl is 0.3 mg/ml. Add the CoCl second to prevent precipitation. This solution must be prepared fresh.
- DAB/CoCl staining solution: add 0.3 % $H_2O_2$ 1:1,000 to the DAB/CoCl presoak solution. This solution must be prepared fresh.
- DAB presoak solution: dilute DAB stock solution (40 mg/ml in water) 25:2000 in PBST. Final concentration of DAB is 0.5 mg/ml. This solution must be prepared fresh.
- DAB staining solution: add 0.3 % $H_2O_2$ 1:1,000 to the DAB presoak solution. This solution must be prepared fresh.
- Blocking solution: 2 % Normal Goat Serum (NGS), in PBST with 1 % DMSO
- Anti-fluorescein Fab-peroxidase (Roche Mannheim)
- Quenching solution: 0.5 ml 3 % $H_2O_2$ added to 2.5 ml absolute methanol

## Method

***Permeablization of embryos:***

***Transfer fixed embryos into distilled water for 2 min (for gastrulae) to 5 min (for 24 h embryos).***

***Remove water (leaving embryos covered).***

***Add ice cold acetone for 3 min (for gastrulae) to 7 min (for 24 h embryos).***

***Remove acetone (leaving embryos covered.)***

***Watching the embryos under a dissecting scope in a spot plate, add water. As embryos begin to swell, gently add back PBST to stop swelling.***

***For embryos older that 2 days: Transfer to quenching solution for 30 min. Afterwards, wash three to five times with PBST.***

***If an antibody staining reaction for endogenous markers is to be done, insert method here.***

***Staining of biotin labeled cells:***

1. Preparation of pre-complexed AB solution: Dilute solutions A and B from the elite ABC kit 1:200 in 1% DMSO in PBST. (For the standard kit, 1:100). Mix this solution 20 min before using (to precomplex the avidin-biotin HRP)
2. Incubate embryos with pre-complexed AB solution for 1 to 2 h.
3. Wash with PBST for 15 min, then 30 min, and then 1 h.
4. Incubate for 15 min in DAB/CoCl presoak solution.
5. Watching under a dissecting scope, develop the embryos with DAB/CoCl staining solution (to a blue black color)
6. Stop the reaction by washing three to five times with PBST.

***Staining of fluorescein labeled cells:***

1. Transfer embryos to blocking solution for 1 to 2 h at room temperature or overnight at 4 °C.
2. Incubate with in anti-FITC (dilute 1:1,500 in block) for 2 to 4 h at room temperature or overnight at 4 °C.
3. Wash with PBST for 15 min, then 30 min, and then 1 h.
4. Incubate for 15 min in DAB presoak solution.
5. Watching under a dissecting scope, stain the embryos with DAB staining solution (to a brown color)
6. Stop the reaction by washing three to five times with PBST.

## Protocol 10. Mounting procedure for stained embryos

### Equipment and reagents

- Ethanol series: 30%, 50%, 70%, 80%, 90%, 100%, 100%
- 2:1 benzylbenzoate and benzyl alcohol
- Permount
- Bridged cover slips: 60x25 mm cover slips with 2 #2 thickness 22x22 mm on each end, glued together with Permount. Make several days ahead.
- 40x24 mm Coverslips

### Method

1. Wash embryos in distilled water.
2. Dehydrate the embryos by washing through the ethanol series, 5 min at each step.
3. Transfer the embryos to a 2:1 mixture of benzyl benzoate and benzyl alcohol, and wait until the embryos sink (about 15 min).
4. Transfer the embryos into a small (about 10 to 20 µl) droplet of the benzyl benzoate and benzyl alcohol solution on a bridged cover slip. Carefully remove excess solution with a tissue.
5. Overlay with Permount, orient the embryos, and then cover with 40x24 mm cover slip.
6. Slide and wiggle cover slip around to adjust orientation of embryo.
7. The preparation is permanent, and lasts several years.

## Procedures for analyzing data.

Describing the histotypes and fates of cells is the most difficult aspect of the analysis of time lapse or transplantation, requiring care and time. For each animal produced, the cells and their histotypes must be recorded at the end of the experiment. This is usually the most time consuming part of the experiment; a transplantation takes a minute or so but its analysis at 30 h takes about 20 min to 45 min, and sometimes much longer.

At each time point, we typically hand record the location and morphology of each labeled cell in the embryo, recording hand notes on a template to help interpret—and find—the images at later dates. This is shown in Figure 8. We also document each group of cells on video or computer files, recording data as separate images for white light and UV epilumination, usually at many different focal planes. Occasionally we will re-check complicated sections using a confocal microscope. Also, pay attention to dead and dying cells. In both lineage and transplantation experiments, dead

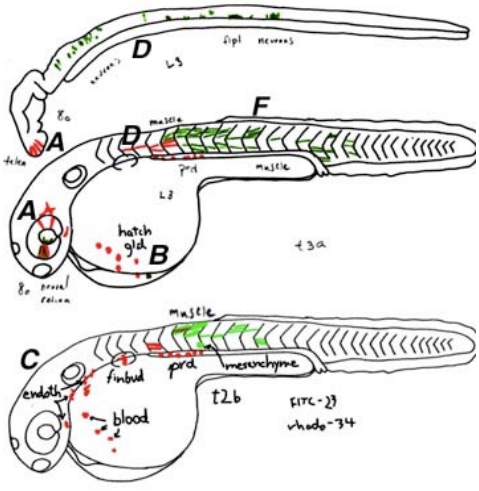



Fig. 8: Documentation of the location and morphology of labeled cells

cells can indicate over injection or photodamage to the cells during the experiment. In transplantation experiments testing cell autonomy, dead cells may be an indication that the mutations are cell lethals.

One minimal strategy for data management is to score only neurons and muscle cells, which together comprise about 50% of the embryo. This data, expressed as a ratio, can reveal subtle changes in fate, especially when measured and averaged over many embryos. For example, in such an experiment, a given distance from the blastoderm margin should give a somewhat reproducible ratio between ectoderm and mesoderm; a change in this ratio, in a mutant or experimental embryo, may indicate changes in fate.

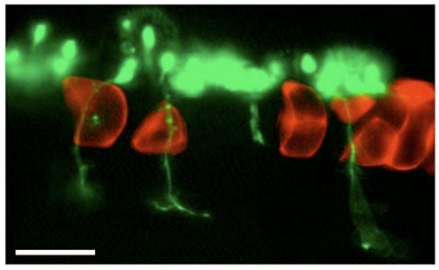


Fig. 9: Stained notochord

Hopefully, a figure must be prepared from the collected data. We have given an number of examples in our figures. Most biotin labeled preparations can be simply photographed onto film or captured using a color video camera. Images captured with black and white cameras are more of a challenge. Typically, a single image will be prepared from a DIC image, and 1 to 3 epiflourescence images. First, we process the individual images to match the black background to the same level, trying to avoid over editing. Then, to fuse these images into one RGB image, we place the rhodamine image into the red channel, the flouresceine image into the green channel and the DAPI image into the blue channel. (Unused channels are filled with a black background for most programs.) The resulting composite image with a black background is often the best image, as shown in Figure 5E or Figure 9. Alternatively, the epiflourescent image is fused or masked over the DIC image, such as in Figures 3, 4E&F and 6.

Good luck.

Table 2. Common Transplantation / Lineage Analysis Solutions.

| | |
|---|---|
| E2 | 15.0 mM NaCl, 0.5 mM Kcl, 1.0 mM CaCl2, 1.0 mM MgSO4, 0.15 mM KH2PO4, 0.050 mM Na2HPO4, 0.70 mM NaHCO3.<br>Make as combined 20x stock, except for BiCarb, which is made as a 200x or 500x stock. (For most uses, BiCarb can be omitted.)<br>pH of final stock should be 7.0 to 7.5. |
| Danieau's | 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES pH 7.1 to 7.3.<br>Make each component as a separate 50x solution. |
| streptomycin / penicillin | 50 µg/ml streptomycin-50 U/ml penicillin.<br>Purchase and store as frozen 1000x solution. Use media within 24 h of preparation. |
| Methyl cellulose | Make as 3% in either water or E2 medium.<br>Dissolve by mixing over several hours while partially freezing, clarify by refrigeration over night; dispense in 1 or 5 ml syringes; refrigerate. |
| MESAB (also called Tricaine or MS322) | 4 mg/ml ethyl -m-aminobenzoate methanesulfonate, 1% $Na_2HPO_4$,<br>pH 7.0 to 7.5; refrigerate<br>For use, dilute 1:20 to 1:100; solution will darken on exposure to light. |
| Petroleum jelly (Vasoline) | Melt in boiling water, pour into the back of 5 cc syringes, and put the plungers back in; dispense through a cut-off 18 gauge needle. |
| Agar/ agarose media | 1.5% agar for lining plastic Petri dishes; 1.5% agarose for molds;<br>0.1% agarose for immobilizing embryos for timelapse.<br>Made in Danieau's, E2, or water.<br>The yolk cell membrane will stick to dryed agar surfaces; this can be corrected by hydrating the plates over night. Check agarose brands before use, the yolk cell sticks to many that are normally used for electorphoresis; this can be corrected by cutting 10 parts agarose:1 part agar. |
| Watch maker forcepts (#5 Dumont) | Use three in normal work. The sharpest, just out of the package with 20µm tips, is the tool for dechorionation and fine manipulations such as removing blastoderms etc. Slightly duller, with 50 µm tips, is a tool for holding the chorion or embryo. The dullest, with 100 µm or more tips, is a good tool for general embryo work such as sorting and cleaning embryos or orienting embryos in methylose. Even on out of the package, touch up forceps with a fine stone under a dissecting scope; all forceps, fine to coarse, must make first contact at the very end of the of the tongs. |
| nylon 'hair' loops | Use lowest test weight fishing line, about 100 to 150 µm diameter, to fashion small 1-2 mm loops at the end of a glass capillary; fix to a glass capillay with glue or wax. |
| Tungstan needles | sharpen a tungstan wire by electrophoresis in a solution of xxx. The needle is attached to the xxx pole of a DC source; graphite or a pencil lead is used as a cathode; fix the sharped needle to a glass capillay with glue or wax. |
| cactus needles | Pick the finest needles from the top of the cactus plant; harden the needles by heating on an incandescent light bulb; fix to a glass capillary with glue or wax. For continued good results, water the cactus plant once or twice a year. |

References
1. Roosen-Runge, E. (1939) *Biol. Bull.*, **77,** 79-91.
2. Kimmel, C. B., Warga, R. M. and Schilling, T. F. (1990) *Development*, **108**(4)**,** 581-94.
3. Stainier, D. Y., Lee, R. K. and Fishman, M. C. (1993) *Development*, **119**(1)**,** 31-40.
4. Woo, K., Shih, J. and Fraser, S. E. (1995) *Current Opinion in Genetics & Development*, **5**(4)**,** 439-43.
5. Woo, K. and Fraser, S. E. (1995) *Development*, **121**(8)**,** 2595-609.
6. Schilling, T. F., Walker, C. and Kimmel, C. B. (1996) *Development*, **122**(5)**,** 1417-26.
7. Melby, A. E., Warga, R. M. and Kimmel, C. B. (1996) *Development*, **122**(7)**,** 2225-37.
8. Felsenfeld, A. L., Walker, C., Westerfield, M., Kimmel, C. and Streisinger, G. (1990) *Development*, **108**(3)**,** 443-59.
9. Streisinger, G., Coale, F., Taggart, C., Walker, C. and Grunwald, D. (1989) *Developmental Biology*, **131(1),** 60-69.
10. Strehlow, D., Heinrich, G. and Gilbert, W. (1994) *Development*, **120**(7)**,** 1791-8.
11. Hammerschmidt, M. and Nüsslein-Volhard, C. (1993) *Development*, **in press**.
12. Melby, A. E., Kimelman, D. and Kimmel, C. B. (1997) *Developmental Dynamics*, **209**(2)**,** 156-65.
13. Girdham, C. H. and O'Farrell, P. H. (1994) *Methods in Cell Biology*, **44,** 533-43.
14. Cambridge, S. B., Davis, R. L. and Minden, J. S. (1997) *Science*, **277**(5327)**,** 825-8.
15. Garcia-Martinez, V. and Schoenwolf, G. C. (1993) *Developmental Biology*, **159**(2)**,** 706-19.
16. Ho, R. K. and Kimmel, C. B. (1993) *Science*, **261,** 109-111.
17. Ho, R. K. and Kane, D. A. (1990) *Nature*, **348**(6303)**,** 728-30.
18. Shih, J. and Fraser, S. E. (1996) *Development*, **122**(4)**,** 1313-22.
19. Oppenheimer, J. M. (1936) *J. exp. Zool*, **72,** 247-269.
20. Sagerstrom, C. G., Grinbalt, Y. and Sive, H. (1996) *Development*, **122**(6)**,** 1873-83.

---

# 7.6 (Additional Protocol) Production of chimeric medaka

(Y. Wakamatsu; modified to Ando & Wakamatsu, 1995)

## Preparation

## Fish

Two medaka (Oryzias latipes) strains different in body color are used (wild-type strain as donor, albino strain as recipient)

## Reagents and Equipments

- 90% calcium- and magnesium-free phosphate-buffered saline (90% CMF-PBS)
- Balanced salt solution for medaka (BSS) (Iwamatsu, 1983)
- BSS supplemented with 100 U/ml penicillin and 100µg/ml streptomycin (BSS+PS)
- BSS supplemented with 2 ppm methylene blue (BSS+MB)

- Wide-mouthed pipet
- Stereoscopic microscope(magnification of 60-70)
- Plastic suspension culture dish (diameter 60mm,SUMILON)
- Fine forceps (INOX No.5)
- Micromanipulator (Eppendorf, NARISHIGE MN-2)
- Microneedle
- Loading capillary
- Degassed water
- Liquid paraffin
- Orientation needle (glass tube with plastic thread at tip)

## Preparation of hatching enzyme solution

Culture embryos in 0.5 ppm methylene blue solution at 26°C and harvest them just before hatching (8-9 days after fertilization). To remove methylene blue, transfer them to distilled water one day before collection.

Put harvested embryos in a homogenizer and add equivalent volume of 90% CMF-PBS. Homogenize embryos on ice and store at 4°C overnight.

Centrifuge the homogenate at 15,000 rpm for 10 min at 4°C.

Dispense the supernatant in 100µl aliquots into 1.5 ml centrifuge tubes and store at -80°C.

## Preparation of agar plates

Embryonic cell transplantation is carried out on an aseptically-prepared agar plate.

Pour sterilized BSS containing 2% agar into a 6 cm plastic dish to a depth of 3 mm.

After the agar hardens, make two V-shaped grooves 1 mm wide and 1 mm deep for orientation of embryos with a sterile razor blade (Figure 5). It is easy to make a groove using a razor blade held by forceps.

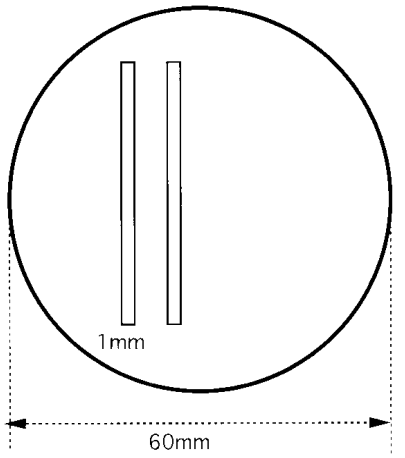



Figure 1: Agar plate. Two V-shaped grooves are made at one side of the agar plate.

## Collection of embryos

Embryos at midblastula stage (Iwamatsu,1994) are used for transplantation. Collect embryos before morula stage because embryos continue to develop during the hatching enzyme treatment.

Scoop females up with a net and collect clusters of eggs from the abdomen. Place egg clusters in the center of a paper towel and roll them pressing gently with another paper towel. Don't press the clusters firmly, as embryos at the early stage are easily injured. The attaching filaments are broken and removed by this treatment, and egg clusters separate into single eggs.

Transfer single eggs to dishes of distilled water. Remove unfertilized (lacking fertilization membrane) and dead eggs. Eggs whose chorion has been dented in the process of removal of attaching filaments return to normal after a short time, but eggs whose blastoderm has become blackish soon die.

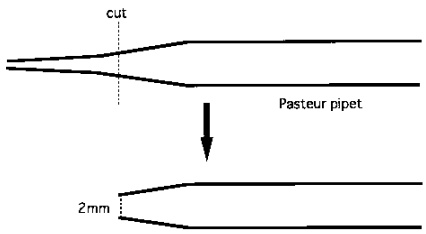



Figure 2: Wide-mouthed pipet. Wide-mouthed pipets are made by cutting pasteur pipets where the inner diameter is 2 mm and fire-polishing the cut end .

## Removal of chorion

Medaka embryos are covered with a 2-layer chorion that has a hard inner layer and a soft outer surface. Glass needles penetrate the hard chorion with difficulty so that operations such as aspiration and injection of blastodermal cells cannot be performed smoothly. Therefore, embryos dechorionated with medaka hatching enzyme are used for transplantation. The hatching enzyme is a protease, which is secreted from the hatching enzyme gland and dissolves the inner layer of the chorion (Yasumasu, et al, 1994). Dechorionated embryos develop normally and at the same rate as embryos with a chorion.

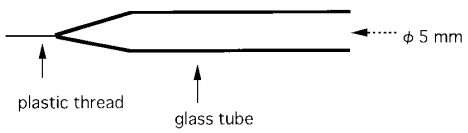



Figure 3: Orientation needle. Orientation needles are made by fixing the plastic thread to the tip of 5 mm o.d. glass tubing.

Arrange eggs in a monolayer in a well of a 24- or 96-well plastic plate. If eggs are piled on top of each other, eggs at the bottom are crushed when the chorion dissolves. Immerse eggs in the hatching enzyme solution and keep at 26°C. Check periodically under a stereoscopic microscope for dissolution of chorion. When the enzyme begins to work, a number of lunar crater-like holes open in the inner layer, and the inner layer soon disappears, generally within 30-60 min. After this treatment, use sterile tools and reagents.

Add BSS+PS gently after the inner layer dissolves.

Pipet gently with a wide-mouthed pipet to float embryos. Draw up floating embryos slowly and transfer gently to BSS+PS in a plastic suspension culture dish. Use this type of dish after this step to prevent dechorionated eggs from adhering to dishes.

Transfer embryos to fresh BSS+PS to remove hatching enzyme.

Pinch the outer layer with a fine forceps sterilized in 70% alcohol and agitate gently it in BSS+PS. The outer layer of the chorion can be easily removed.

Transfer dechorionated eggs to fresh BSS+PS with a wide-mouthed pipet. Don't make dechorionated eggs come into contact with air. When dechorionated eggs are drawn up with air bubbles, they collapse instantly.

Incubate embryos at 26°Cuntil they develop to midblastula stage, and store them at 4°C to stop development until use.

## Preparation of donor ES cells for chimera formation

During handling ES cells in multiwell plates, avoid complete removal of fluid in order to prevent cell death from drying. Primary ES cell cultures are used for production of chimeras showing wild-type pigmentation, while MES1 cells are used for observing their distribution into other cell lineages by GFP transgene expression.

1. Add 1 ml of 0.2 µm-filtrated PBS to primary blastula-derived cell cultures or MES1 line cells in a 12-well. Do not remove medium before!
2. Partially remove fluid, so that the cells are still covered in fluid.
3. Repeat steps 1 & 2 twice: wash away debris as well as FBS for trypsinization.
4. Add 0.5 ml trypsin (Gibco) diluted (1x) in PBS, incubate at room temperature for 3-5 min. Carefully monitor under microscope and stop the reaction when cells become round: partially remove fluid and add 0.5 ml medium immediately.
5. Make single cell suspension by pipetting. Transfer to 1.5-ml tubes.
6. Spin at 5000 rpm for 2 min. Aspirate fluid and resuspend cells in 0.2 ml ESM1 medium by pipetting.
7. Recover cells at 28°C for 1-3 h. (during this step, move to work with host embryos)
8. Spin again as above, remove medium, wash cells 3x with TM1, 200 µl each time.
9. Resuspend cells in 20 µl TM1 for transplantation. 2 µl is suuficient for 50 embryos.

## Transplantation

Transplantation is carried out under a stereoscopic microscope with a micromanipulator .

1. Draw degassed water up in a loading capillary. Insert the loading capillary into a microneedle from the back and fill the microneedle with degassed water. Don't allow air bubbles to enter the microneedle.

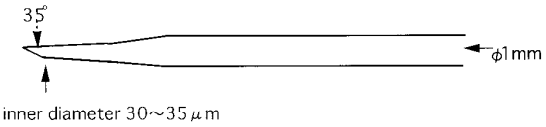



Figure 4: Microneedle. Microneedles are prepared by pulling 1 mm o.d. glass capillary tubing with a puller (e.g. Narishige PN-3). The tip of microneedle is sharpened with a needle sharpener (Narishige EG-4). The inner diameter at the opening is 30µm and the angle is 35°.

2. Place the microneedle filled with degassed water in the needle holder of the micromanipulator. Draw liquid paraffin up to 10-15 mm from the top of the microneedle. Liquid paraffin prevents intense pressure changes in the needle.

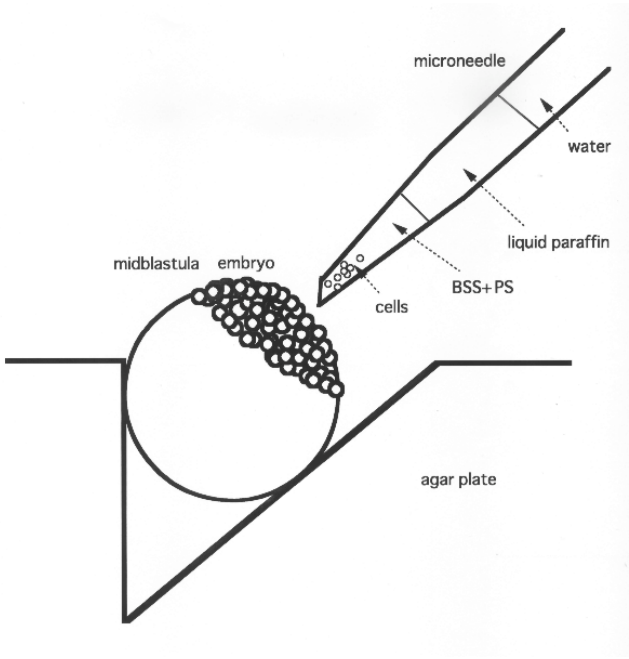



Figure 5: Scheme of transplantation. Approach of the microneedle to an embryo from the side without grooves.

3. Pour BSS+PS onto agar plate. Place midblastula-stage donor embryos in one groove and same-stage recipient embryos in the other. With an orientation needle, arrange embryos with blastoderms facing the microneedle(Figure 7A, B).
4. Puncture the donor blastoderm with microneedle and slowly draw up donor cells from deep in blastoderm core.
5. Pull microneedle out of donor blastoderm and wait a few minutes while aspirated cells fall down and gather at the tip of the microneedle.
6. Insert microneedle into the recipient blastoderm and inject harvested cells slowly.

## Culture and observation of transplanted embryos

Incubate transplanted embryos in BSS+MB at 26centidegree.

Change BSS+MB every 3 days.

Remove dead embryos. Dead embryos stain blue with methylene blue. Handle the dish gently because dechorionated embryos are sensitive to mechanical shock. Transplanted embryos develop at nearly the same speed as control embryos.

After 2-3 days, melanophores appear on the yolk sac, head, eyes, and trunk of transplanted embryos . Appearance of melanophores is a sign of successful formation of chimeras.

After 7-10 days , embryos begin swimming , which shows that embryos have hatched.

See Wakamatsu, et al. (1993) for description of general methods.

## References

Iwamatsu, T. (1994). Stages of normal development in the Medaka *Oryzias latipes*. Zool. Sci. 11, 825-839.

Wakamatsu, Y. et al. (1993). Generation of germ-line chimeras in medaka. Mol.Mar.Biol.Biotech. 2, 325-332.

Yasumasu, S. et al. (1994). cDNAs and the genes of HCE and LCE, two constituents of the Medaka hatching enzyme. Develop. Growth & Differ. 36, 241-250.

# 8. CELL CULTURE

## 8.1 Isolation and long-term cultivation of medaka blastula cells

(contributed by Y. Hong)

Preparations (in a tissue culture hood)

1. Coat 24-well plates with 0,1% gelatin solution (0,5 ml/well) for 30 min.
2. Aspirate gelatin solution and allow plates to air-dry for 2h.
3. Add 1ml of ESM4 medium per well and pre-warm in a 28°C incubator.
4. Put a dissecting microscope into a culture hood. Sterilize by 70% ethanol & UV treatment for 20 min.

### Isolation and primary culture

1. Collect clusters of eggs in a 6-cm Petri dish with ERM within 2 h postfertilization.
2. Roll the eggs in the dish with a finger to remove larger hairs.
3. Wash and transfer fertilized (transparent) eggs to a new dish with ERM (Embryo Rearing Medium).
4. Incubate at 26°C until 6h post fertilization.

The following steps are done in a tissue culture hood

5. Transfer embryos to 6-well plates
6. Treat embryos with 10 ml 0,5% bleach for 1 min.
7. Wash embryos with PBS 4-5 times.
8. Transfer 20-30 embryos with a drop (300-400 µl) of PBS to the middle of a 6-cm dish.
9. Poke the chorion and yolk with fine forceps under a dissecting microscope. Let stand for 5 min (The yolk material will flow out).
10. Replace half of PBS in the drop 3-5 times to remove yolk material.
11. Press the chorion to release cells through the hole. Remove chorion and hairs.
12. Make a cell suspension by pipetting. Swirl the suspension by pipetting in one direction at the marginal region of the drop. Let stand for 5 min for the cells to settle down in the middle of the drop.
13. Suck up 200-300µl supernatant carefully, without disturbing the cells. Add 200-300µl PBS. Pipette and swirl the drop (Keep the drop in a diameter not larger than 1 cm).
14. Repeat 4-5 times.
15. After final washing step, suck up the cells with 100µl PBS and seed evenly in 1 ml ESM4 medium in a 24-well plate pre-warmed at 28°C.
16. Culture the cells in a 28°C incubator *WITHOUT* $CO_2$.
17. After 1 day, change 800 µl medium and culture for 3-4 days. Observe daily.

Hong, Y., Schartl, M.: Establishment and growth responses of early medakafish (*Oryzias latipes*) embryonic cells in feeder layer-free cultures. Mol. Mar. Biol. Biotechnol 5, 93-104, 1996.

Hong, Y., Winkler, C., Schartl, M.: Pluripotency and differentiation of embryonic stem cell lines from the medakafish (*Oryzias latipes*). Mech. Dev. 60, 33-44, 1996.

---

## 8.2 Transfection of medaka ES cell cultures

(contributed by Y. Hong)

Several protocols are effective for gene transfer into medaka ES cell cultures. Electroporation and GeneJuice Transfection Reagent (70967-3; Novagen) reproducibly give rise to a high efficiency (25-40%), while liposomes are inefficient. Here one stable medaka ES cell line, MES1, will be transfected with a GFP expression vector by using GeneJuice Transfection Reagent.

1. Seed 1 ml of MES4 cell suspension ($10^5$/ml ESM1) in gelatin-coated 12-well plates one day before so that cell density will be 70% confluency at transfection.
2. Prepare transfection mix by combining:
   (100 µl mix containing 2 µg plasmid DNA for 1 ml medium in a 12-well)

   A: 40 µl pure DMEM medium (no antibiotics, no serum)
   10 µl GeneJuice Transfection Reagent
   mix by pipetting gently
   B: 48 µl pure DMEM medium (no antibiotics, no serum)
   2 µl pCVpf DNA (µg/µl)
   mix by pipetting gently

   Add A to B, mix by pipetting. Incubate in cell culture hood for 5-10 min
3. Drop the mix to cells.
4. Two days later, the cells are checked by fluorescent microscopy: 5-20% are green due to GFP. The cells are ready for drug selection or transplantation into blastulae.

---

## 8.3 Transplantation of cultivated medaka cells into blastula embryos

(contributed by Yunhan Hong)

### Preparation of donor cells at the log phase

1. Day 0: Seed $10^6$ MES1 (Medaka ES) cells expressing eGFP in 2 ml ESM4 medium in a 6-well plate and culture at 28°C.
2. Day 1: Change the medium and culture at 28°C.
3. Day 3: Aspirate the medium, wash 3x with PBS. Incubate cells with 1 ml PBS at RT for 10 min. Pipette vigorously and transfer cell suspension to 1.5 ml tube. Spin at 5000 rpm for 2 min. Resuspend cell pellet in 1 ml PBS. Spin again. Wash twice with 0.5ml transplantation medium (TM1). Resuspend cells in 10-20µl TM1. Cells are now ready for injection.

## Preparation of recipient embryos

Collect eggs shortly after fertilization. Remove the attachment filaments by finger-rolling in a 10-cm dish with ERM. Wash 3x with water.

Transfer eggs in a droplet of $H_2O$ to the center of a dry 3-cm dish. Remove residual $H_2O$. Treat in 200µl proteinase K (20mg/ml) at 28°C for 1-2 h. Wash 3x with $H_2O$.

Transfer embryos with $H_2O$ into a 3-cm non-adhesive dish in such a way that embryos are arranged in a drop not larger than 1 cm in diameter. Remove excessive $H_2O$ so that all embryos are just immersed but never exposed to air. Add hatching enzyme (HE) to the drop to cover all embryos (300-400 µl). Position embryos in a single layer. Be sure that all embryos are submerged. Incubate at 28°C for 2-3 h.

Carefully put under a binocular for observation: chorion is fragmented or completely digested. Pipette BSS-1%PEG and gently add to the drop: 5 ml/dish. Remove chorions from all HEALTHY embryos by using a pair of fine forceps. Transfer dechorionated embryos with a wide-opening plastic pipette into a 3-cm non adhesive dish with 5 ml BSS-1%PEG. Now the embryos are ready for injection.

***Note:*** Be careful when transferring dechorionated embryos, which are very fragile. Avoid air bubbles which will destroy embryos. Avoid direct contacts of embryos with solution surface, which also results in embryo collapse. During transfer, embryos should be floated in the middle of the narrow part of a plastic pipette which is fully filled with BSS-1% PEG. Insert the tip down to the solution in the dish, push the solution slowly and gently out from the pipette.

## Transplantation

(All transplantation steps are performed under sterile conditions (if possible!))

### Preparation of transplantator or needle holder

1. Wash transplantator thoroughly with ethanol and rinse with Millipore water
2. Fill device with light mineral oil (Sigma # M3516) and place all components together
3. Fill a transplantation needle with TM1 avoiding air bubbles; insert needle into device
4. Place transplantator on top of a micromanipulator
5. Break needle tip
6. Pipette 5µl droplet of donor cell suspension into a sterile petri dish; bring needle tip into the suspension and carefully suck up cells into the needle
7. Remove needle from the suspension and let the cells sink down in the needle tip

### Orientation and injection of recipient embryos

1. Transfer dechorionated recipient embryos to an agarose ramp or plastic devices (also used for oocyte injection) containing BSS-1%PEG (12-15 ml per 6-cm dish).
2. Make an opening approx 30 µm in diameter at the tip of a needle and put the needle into a 1.5-ml microfuge tube filled with TM1, the sharp tip facing up: the needle is loaded by capillary force. Connect the needle to the micromanipulator, remove the air from the needle. Load 2-3 µl of the MES1 donor cells into a tiny drop on the cover of a 3-cm dish. Insert the needle to the cell drop and suck cells by a gentle negative pressure.
3. Carefully position embryos using an orientation needle (do not contact embryos with the sharp tip, but the side of the tip), so that the cell mass (milky) faces the needle tip.
4. Insert needle into blastula stage recipient and inject app. 50 cells into the center of the blastula
5. Remove injection needle out the injected embryo as quickly as you can.
6. Transfer embryos carefully from injection device to Petri dish (non adhesive) filled with BSS-1%PEG.
7. Incubate at 18°C the first day, then at 28°C the following days. Change medium twice a day; remove dead embryos

Observation: after successful transplantation, green cells should be visible here or there in the cell mass. After one day, green donors are seen in many different areas: head, trunk, somites, yolk sac. On day 3, the donor cells are in the heart, circulation, eye etc.

## Material needed

- Wide-mouthed pipette
- Stereoscopic microscope
- Plastic suspension culture dishes (bacterial)
- Fine forceps (INOX 5)
- Micromanipulator (Eppendorf)
- Microneedles
- Loading capillary
- Orientation needle
-

## Buffers and solutions

### ESM4 (for blastula cell culture)

| | |
|---|---|
| DMEM | 13 g/l |
| Glucose | 4.5 g/l |
| Hepes | 20 mM |
| Streptomycin/penicillin | 50 U/ml/ 50/µg/ml |
| Non-essential amino acids | 1 mM |
| Na-pyruvate | 1 mM |
| Na-selenite | 2 nM |
| Gutamine | 2 mM |
| 2-mercaptoethanol | 100 µM |
| ES-FCS | 15% |
| Rainbow trout serum (self made) | 1% |

| | |
|---|---|
| bFGF; human recombinant | 10 ng/ml |
| Medaka embryo extract (self made) | 1 embyo/ml |

pH 7.6

filter through 0.2 µm filter

### TM1 buffer (for cell transplantation)

| | |
|---|---|
| NaCl | 100 mM |
| KCl | 5 mM |
| Hepes | 5 mM |
| Phenol red | 2 µg/ml |

pH 7.0

filter through 0.2 µm filter

### Proteinase K

20 mg/ml in water

### BSS (for cell culture)

Solution A (500ml):

| | |
|---|---|
| NaCl | 65g |
| KCl | 4g |
| $MgSO_4 \times 7H_2O$ | 2g |
| $CaCl_2 \times 2H_2O$ | 2g |
| Phenol red | 5mg |

autoclave

Solution B:

| | |
|---|---|
| $NaHCO_3$ | 5% |

filter through 0.2 µm filter

dilute 25ml solution A with 475ml of distilled water and add 1ml of solution B

### BSS-1% PEG

| | |
|---|---|
| PEG20000 in BSS | 1% |
| Streptomycin/penicillin | 100 µg/U/ml |

Sterile filter through 0.2 µm filter

## References

Hong, Y., Winkler, C., Schartl, M. (1998) Proc. Natl. Acad. Sci. 95, 3679-3684.

Hong, Y., Winkler, C., Schartl, M. (1998) Dev. Genes Evol. 208, 595-602.

# 8.4 (Additional protocol) Primary culture of blastula cells in medaka

(Y. Wakamatsu)

## Materials

- Medaka hatching enzyme solution[2]
- Medaka balanced salt solution supplemented with antibiotics (BSS + PS)[2]
- Culture medium[3]: Culture medium is prepared by modyfying Robertson´s methods for culture of mouse embryonic stem cells[4], that is, Dulbecco´s modified Eagle medium supplemented with 15% fetal calf serum, 5% carp serum, 100U/ml penicillin, 100µg/ml streptomycin, non-essential amino acids[5], nucleosides[6], 2-mercaptoethanol[7], $10^3$ U/ml leukemia inhibitory factor, 20ng/ml basic fibroblast growth factor, and 25mM Hepes (pH 7.3)
- Trypsin-EDTA solution[3]: Calcium- and magnesium-free Dulbecco´s phosphate buffered saline containing 0.25% trypsin and 0.04% EDTA is sterilized by filtration.

## Methods

### Collection of embryos

Medaka spawns every day throughout the year under a controlled condition of temperature (26°C) and lighting (14 hours light and 10 hours dark).

1. Collect 50 to 60 eggs in the morula stage[8] from females belly.
2. Remove the attachment filaments of eggs by rolling them on a piece of paper[2].
3. Rinse the eggs twice in water.

### Preparation of blastoderms for culture

1. Remove the chorion by using medaka hatching enzyme solution[2]. All procedures are done under sterilized conditions after this step.
2. Rinse the dechorionated embryos twice in BSS+PS in a 60mm plastic dish for suspension culture.
3. Suck the embryos gently into a pasteur pipet. Yolk of the embryos is broken by this treatment.
4. Remove yolk fragments attaching to the blastoderms by fine forceps.
5. Rinse the resulting cell masses of blastoderms twice in BSS+PS.

### Culture of blastoderm cells

1. Divide each cell mass into several smaller fragments by forceps.
2. Centrifuge the fragments at 500 rpm for five minutes
3. Resuspend them in 500µl of the culture medium and inoculate them in a single well of a six-well plate.
4. Inoculate the plate at 28°C in air.
5. The fragments attach to the substratum one to four days after inoculation. Replace half of the medium in the well with fresh medium every two to three days.
6. One week after inoculation, the attached fragments exhibit an active outgrowth. Dissociate the fragments into smaller fragments and single cells by trypsinizing them with trypsin-EDTA solution and pipetting.
7. Inoculate the resulting smaller fragments and single cells in a single well of a six-well plate.
8. Replace half of the medium in the well every two to three days.
9. When the cultures have grown, subculture them in larger dishes by routine culture methods.

### Notes

[1] Wakamatsu, Y., Ozato, K., Sasado, T. (1994). Establishment of a pluripotent cell line derived from a Medaka (Oryzias latipes) blastula embryo. Mol.Mar.Biol.Biotec. 3, 185-191.

[2] See the protocol "Production of chimeric medaka (Oryzias latipes)"

[3] Osmotic pressure of all solutions used for culture is adjusted to 90% of that in mammalin cell culture by diluting them with double distilled water.

[4] Robertson, E.J. (1987). Embryo-derived stem cell lines. In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. Robertson, E.J. 8ed.), Oxford, IRL Press, pp. 71-112.

[5] 100x non-essential amino acids solution (Gibco)

[6] 100x nucleosides stock solution: Dissolve 80mg adenosine, 85mg guanosine, 73mg cytidine, 73mg uridine, and 24mg thymidine in 100ml double distilled water at 37°C and sterilize by filtration.

[7] 100x 2-mercaptoethanol stock solution: Dissolve 7µl of 2-mercaptoethanol in 10ml double distilled water and sterilze by filtration.

[8] Iwamatsu, T. (1994). Stages of normal development in the medaka Oryzias latipes. Zool. Sci. 11: 825-839.

---

## 8.5 Fluorescence activated cell sorting (FACS)

(contributed by Felix Loosli)

Flow cytometry is a method for quantitating components or structural features of cells, primarily by optical means. Although measurements are made on one cell at a time, several thousands of cells can be processed in a few seconds. Cells may be alive or fixed at the time of measurement, but must be in monodisperse (single cell) suspension. They are passed single-file through a laser beam by a continuous flow of a suspension stream. Each cell scatters some of the laser light and also emits fluorescent light excited by the laser. The cytometer typically measures several parameters simultaneously for each cell:

- Forward scatter intensity -> approximately proportional to the cell diameter
- Orthogonal scatter intensity -> approximately proportional to the quantity of granular structures in the cell
- Fluorescence intensities at several wavelengths

Fluorescence intensities can be measured at several wavelengths simultaneously for each cell. In our experiment, embryos of transgenic lines that express green fluorescent protein (GFP) will be dissociated and sorted into GFP expressing and non-expressing cells by FACS. This application can be very useful in combination with tissue and/or stage specific GFP expression to enrich for a cell population of interest from wild type and mutant embryos. Since live cells can be sorted, the enriched fraction can be used for a wide variety of applications such as gene expression studies by RNA isolation or as cell culture material.

## Protocol:

1. Collect embryos roll them in PBS (or on filter paper) and wash them several times in PBS.
2. Transfer them to a 6 cm plate filled with TM-1% PEG ($Ca^{2+}$/$Mg^{2+}$ free medium).
3. Open the chorion with a pair of forceps and pull the embryo out of the chorion. (this will disrupted the yolk cell). Remove the debris of the chorion from the medium with a pipette (blue tip and cut off tip).
4. Transfer the embryos to 2-3 ml of prediluted trypsin solution (0.5 g/l). The embryos are then triturated at room temperature through a narrow bore pipette tip (yellow) until they are dissociated (check under microscope).
4. Transfer cells to a 15 ml Falcon tube add 10 ml TM-1 medium +10% sheep serum and centrifuged 5 min at 1000 g and discard the supernatant.
6. Repeat this step once and resuspend the cells in 500 µl TM-1
7. This suspension is filtered through a cell strainer (70 µm, Nylon, FALCON 2350) and the residues on the filter rinsed with another 500 µl of TM-1.
8. Cells are kept on room temperature while waiting for the FACS. Sorted cells are captured in tubes containing 1ml TM-1 and harvested by centrifugating 7 min at 1000 g.
9. Alternatively cells can be captured diretly in Trizol for immediate RNA preparation.
10. For preparation of specific cDNA use the Clontech kit (smart) and follow the instuctions of the supplyer
11. You may alternatively use the RNA for subtractive approaches (Clontech PCR select).

## Material needed

6 cm dish

forceps

15ml Falcon tube

cell strainer 70 µm, Nylon, FALCON 2350

sample tube

collection tube

Trypsin-EDTA 1x solution (Gibco 25300-054)

## Buffers and solutions

TM1 buffer:

| | |
|---|---|
| NaCl | 100 mM |
| KCl | 5 mM |
| Hepes | 5 mM |
| PEG 20000 | 1% |

pH7.0, filter through 0.2 $\mu$m filter

TM-1 buffer + 10% sheep serum

# 9. DATABASE RESOURCES

## 9.1 Internet Information Resources: ZFIN Database

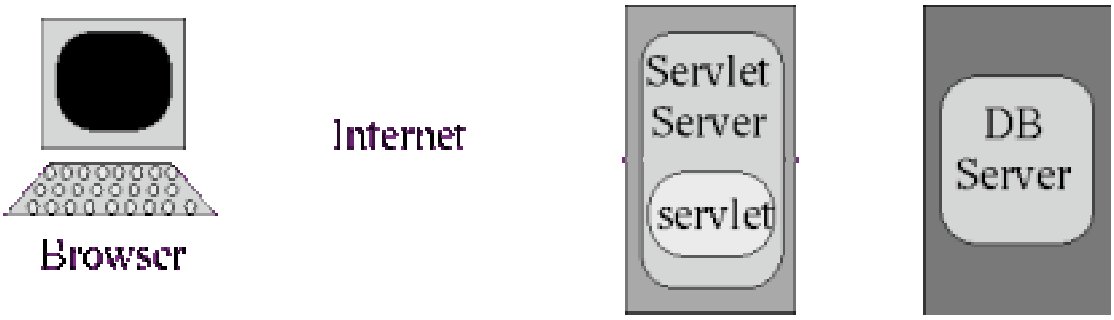
(M. Westerfield)

The specific exercises will be passed out during the course. To prepare ahead of time, read through the on-line information at:
http://zfish.uoregon.edu/zf_info/dbase/db.html

Particularly read:
http://zfish.uoregon.edu/ZDB/PAPERS/sdb/dbs.html

## 9.2 From an experiment to a web accessible database

(Thorsten Henrich)



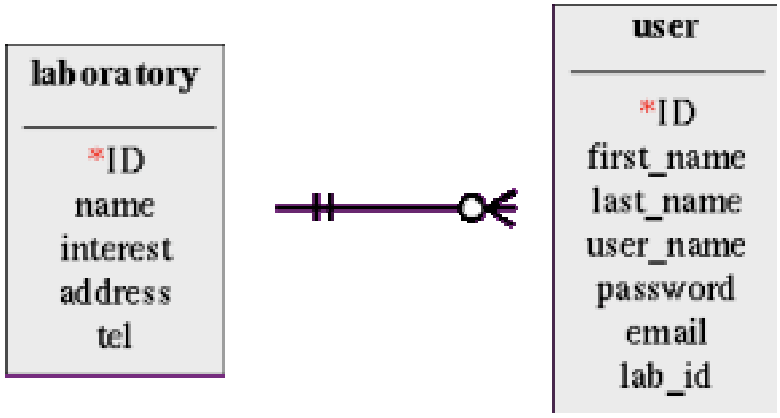

In this practical course we will implement our own small web accessible database. The purpose of the database can be freely chosen depending on your interests. We will use a relational database and start to create the structure of the database, fill it with data and search it using SQL commands. In the second part, we will learn how to access the database via internet using Java servlets displaying the results in a web browser.

### Database

#### Creating the Database structure

***Drawing an ER diagram***

We start drawing entity-relations (ER) diagrams of the database. The important thing is to identify entities and their attributes. Imagine you have a list of users, to whom you want to give access to the database. In this case an user is an **entity** which can be described by the **attributes** first_name, last_name, user_name, password, laboratory. The laboratory itself can be regarded as an entity as well. The ER diagram for this two-tabled database would look like this.



The table user has 7 columns (ID, first_name...). The column lab_id connects the two tables with each other. It is the foreign key to the tables laboratory. The two tables above have a one to many relationship. One lab can have many users and each user is member of only one lab.

### *Creating the database*

In the next step we will translate the ER diagram into a relational database model. This basically means to find the proper SQL commands to create the database tables. Here we will learn more about the SQL commands create table, drop table and alter table. To create the two tables shown above the following commands have to be executed.

```
> create table laboratory (id smallint not null, name varchar(20), interest varchar(30), address
  varchar(30), tel varchar (15), primary key (id))
> create table user (id smallint not null, first_name varchar(10), last_name varchar(10), user_name
  varchar(8), password varchar (8), email varchar(60), lab_id smallint, primary key (id), foreign
  key lab_id (id) references laboratory on update restrict on delete restrict)
```

Because table user depends on table laboratory it can only be created if table laboratory exists already. To check if the table has been created properly type this:

```
> describe select * from laboratory
```

One can delete a table from a database by entering the following command

```
> drop table laboratory
```

or change it by:

```
> alter table laboratory add column url varchar(128)
```

## Manipulating Data

Once the structure of the database has been established, the database needs be filled with data. First, we will learn how to do this 'by hand' on the command line. Here we will use the SQL commands insert, update and delete.

```
> insert into laboratory values (10, 'JST', 'Medaka', 'Kyoto', '494-6380')
> insert into user values (100, 'Thorsten', 'Henrich', 'henrich', '1616', 'henrich@dsp.jst.go.jp', 'JST')
> update user set password='1617' where user_name='henrich'
> delete from user where id=100
```

## Retrieving Data

Here you learn how to search the database using the SQL command select. The following command selects all columns (*) with ID 100.

```
> select * from user where id=100
```

Combining data from two tables:

```
> select user_name from laboratory, user where tel like '%6380%' and laboratory.id = user.lab_id
```

## Accessing the Database Through the WWW.

### Editing a HTML form

HTML forms enable users to submit data from a browser to a server. An HTML form enables us to parse variables to a Java servlet, which uses this information to communicate with the database. A simple example is a login form.

```
<html>
<head> <title> Login </title> </head>
<body>
```

```
<form METHOD="post" ACTION="/AccessDataServlet">
    User: <INPUT type=text size=10 name=user>
    Password:<INPUT type=password size=10 name=password>
    <INPUT type=submit name=submit value="Login">
</form>
</body>
</html>
```

The INPUT, SELECT and TEXTAREA tags offer various other ways to fill out HTML forms, e.g. check boxes, radio buttons, select lists. You will get a sample HTML form, which contains all possible input tags and we will adjust this file to our purpose.

## Java servlets

Java servlets are written in the Java programming language. This means they have to be compiled using the Java compiler javac.

The comand:

```
javac myProgram.java
```

results in the generation of the Java class file myProgram.class. The Java compiler will complain if your source (myProgram.java) has errors. The templates for two servlets will be provided. One to submit data to the database, the other to search the database. Together we adjust these templates to receive the data sent from our HTML form and to send the proper commands to the database .

### *Getting the Parameters from the HTML form*

In 2.1 you learned how to send data from a browser to a server using an HTML form. Here you learn how to receive this data and handle it.

```
String   user =       request.getParameter("user");
String   password =   request.getParameter("password");
```

### *Talking to the database*

Here you learn how to establish a connection from a servlet to the database using Java database connectivity (JDBC), how to execute SQL statements and process the result set. An example for a password check is shown below.

```
boolean CheckAccess (String user, String password) {
 try {
   Connection con = DriverManager.getConnection("jdbc:db2:dbName",
"dbUser", "dbPass");
   String       command = "select password from user where
user_name='"+user+"'";
   Statement    stmt = con.createStatement();
   ResultSet    rs = stmt.executeQuery(command);
   while (rs.next()){
     if (rs.getString("password").equals(password) ) { return true; }
   }
   rs.close();      stmt.close(); con.close();
 } catch (SQLException e) {e.printStackTrace();}
 return false;
```

```
}
```

### *Response to the Browser*

Once you receive the result set from the database, you have to inform the user about the result, which means you have to send a response to the browser.

```
PrintWriter        out = response.getWriter();
String    user =   request.getParameter("user");
out.println  ("Hello  "+user+"<br>");
```

## Links:

### Servlet Engines

tomcat http://jakarta.apache.org/tomcat/ (free)
WebSphere http://www-3.ibm.com/software/webservers/appserv/

### Servlet Tutorial

http://java.sun.com/j2ee/tutorial/1_3-fcs/doc/Servlets.html

### Java

Java http://java.sun.com/
Java API 1.4 http://java.sun.com/j2se/1.4/docs/api/index.html

### HTML

German and French http://selfaktuell.teamone.de/
NCSA: A Beginner's Guide to HTML
http://www.ncsa.uiuc.edu/General/Internet/WWW/HTMLPrimer.html

### Databases

IBM DB2 http://www-3.ibm.com/software/data/db2/
Oracle http://www.oracle.com/
mySQL http://www.mysql.com/ (free)

### Biological Databases

The Molecular Biology Database Collection: 2002 update:
http://nar.oupjournals.org/cgi/content/full/30/1/1/DC1

## Literature:

Bioinformatics:
Developing Bioinformatic Computer Skills, Gibas and Jambeck, O'REILLY
Servlets:
*Java Servlet Programming*, Jason Hunter, O'REILLY

Java:

*Java How to Program,* Deitel&Deitel, Prentice Hall

*Understanding SQL and Java Together*, M. Eisenberg, Morgan Kaufmann

Databases/SQL:

*A Relational Model of Data for Large Shared Data Banks* E. F. Codd, Communications of the ACM, Vol. 13, No. 6, June 1970, pp. 377-387.

http://www.acm.org/classics/nov95/

*Relational Database Design Clearly Explained* JL Harrington, AP Professional

*SQL the Complete Reverence*, G. Weinberg, McGraw-Hill

*SQL for Dummies*, Allan G Taylor IDG Books Worldwide

*SQL for Smarties,* Joe Celko, Morgan Kaufmann

LINUX:

LINUX in a Nutshell, O'REILLY

# APPENDIX

Embryo Production By In Vitro Fertilization

(Source: C. Walker and G. Streisinger; modified W. Driever)

Overview of in vitro fertilization:

Large numbers of synchronously developing embryos can be obtained by in vitro fertilization. Gametes are expressed from breeding adults by gentle pressure. The sperm are maintained in Hank's saline. The gametes are mixed together in a petri dish. When water is added to the egg- sperm mixture, fertilization takes place very rapidly in 20 to 60 seconds. After 1 minute, the sperm are no longer active. The clutch of embryos is fertilized essentially synchronously and develops synchronously during the early cleavage stages if maintained at constant temperature

1. Set up "crosses" between 1 male and 2-3 females each in breeding traps during the late afternoon of the day before beginning the in vitro fertilization.

("Male induction" of oocyte maturation)

2. Precisely at "dawn" , separate males and females into two tanks. For the females, collect sperm from the males.

a. Anesthetize the male fish by immersion in MESAB (see RECIPES). Gill movements should slow down, They can be lifted out of the anesthetic with a plastic spoon

b. Rinse in fish water, and place belly up in a slit in a damp sponge.

c. Gently blot the genital region with a Kimwipe so that no water is present (very important, because water activates sperm)

d. Stroke the sides of the fish gently but firmly with smooth (Millipore) forceps.

e. As the milky sperm come out of the genital pore, collect them in a microcapillary using gentle suction.

f. Pool the sperm from several males in ice-cold, full-strength Hank's saline. For each 1 µl of sperm, use 50 µl Hanks. Sperm from 5-10 males is adequate for fertilization of several hundred eggs. Sperm in cold Hank's will continue to fertilize eggs efficiently for up to 90 minutes. "Eyeball" the concentration of sperm, collecting enough to make a cloudy suspension.

We often prefer to use the "I-buffer" for sperm storage (116 mM NaCl, 23 mM KCl, 6 mM $CaCl_2$ , then add 2 mM $MgSO_4$ , 29 mM $NaHCO_3$ and 0.5% fructose; pH to 7.2 and filtered through 0.22 micron filter)

3. As early as possible in the morning, start collecting eggs.

a. Anesthetize a female in MESAB

Care should be taken not to leave fish in MESAB for too long. Sometimes fish can be revived from too long exposure to MESAB by irrigating their gills with water. Also, extra care should be taken not to damage the gills. Handling fish "front-to-back" when using spoons and spatulas is helpful. One characteristic of sick fish is that their gills hemorrhage easily and the fish die after exposure to MESAB.

b. Rinse in fish water and blot "damp-dry" on a paper towel. Excess water will swell the eggs and prevent fertilization

c. Place the female in a 35 mm plastic petri dish and with damp (not wet) fingers, press gently but firmly on the belly. If she is prepared to lay eggs, they will come out quite easily

d. Gather the eggs with a spatula and return the female to water. Good eggs are a yellowish, translucent color, whereas eggs that have remained in the female too long are white and watery. To ensure getting good eggs, collect them during the first 90 min after "dawn"

4. Fertilize the eggs

a. Add 30-50 µl of the sperm suspension in Hank's to the eggs

b. Mix gently

c. Add about 0.5-0.75 ml of Fructose-eggwater: (0.5 % Fructose in eggwater) and after 1-2 minutes, add 2 mls more Fructose - egg water. The actual time of fertilization is when the first water is added to the eggs. Typically, 60-90% of the eggs are fertilized in an in vitro cross. Depending on health and unknown factors, females will produce.

Detailed protocol for in vitro fertilization

Place on bench afternoon before squeeze

- 2 paper towels.
- finger bowl or large petri dish for rinsing fish
- 200 ml beakers of MESAB diluted
- 50 µl wiretrol
- spatula or 2, if dividing clutches
- plastic spoon
- "Sharpie" marker

Prepare for O/N storage in refrigerator

- 200 ml Fructose-eggwater: 0.5 % Fructose in eggwater at 28°C
  (egg water is 0.03% Instant Ocean Salt mix)
- Hank's Premix solution, see "Recipes".
- MESAB

Morning of the Squeeze

1. Get ice in the ice bucket.
2. To pre measured sodium bicarbonate (0.35 g), add 10 ml $dH_2O$.
3. Measure 9.9 ml of Hank's Premix solution into a clean test tube with a screw cap.
   a. Add 0.1 ml fresh bicarbonate solution to the Hanks Premix.
   b. Put the Hank's completed solution on ice.

Squeezing Males

Materials Needed:

MESAB solution in two 250 ml beakers

Stereo microscope
Sperm collection apparatus
20 ml beaker of Hank's solution
Finger bowl with fish water
Lamp
Kimwipes

1. Estimate the number of egg clutches to be obtained.
2. Measure 0.05 ml of Hank's for every clutch of eggs anticipated.
3. Put the measured Hank's a 1.5 ml microcentrifuge tube and put in ice.

Procedure

1. Remove two fish from the plastic holding container with net and place into the 250 ml beaker containing the MESAB solution. Repeat this for the other 250 ml beaker.
2. When gill movement has slowed, remove one fish with the plastic spoon.
3. Rinse this fish in the water in the finger bowl and place it upside down in the small sponge in the plastic dish.
4. Gently wipe the region of the anal fin with the corner of a Kimwipe.
5. Place the dish with the fish under the objective of the microscope, with the light illuminating the fish, especially the region of the anal fin.
6. With the capillary tube of the sperm collecting apparatus, gently push aside the anal fins to expose the anus.
7. Using the forceps gently squeeze the sides of the fish at a point just anterior to the anal fins, collecting the sperm with the capillary tube. When finished return the fish to the finger bowl or recovery container.
8. When you have collected sperm from 2-3 fish, add them to the Hanks solution in the small test tube in the ice bucket.
9. Repeat until you have collected the required amount of sperm.
10. Keep the small test tube with the sperm and Hank's solution in the ice. This helps prolong the viability of the sperm.

Squeezing Females

Materials Needed:

MESAB solution in two 250 ml beakers

Sperm in Hank's solution

50 mm petri dishes

10-200 µl pipettor

Egg Water

2-1 ml pipettes and a 25 ml pipette in a liter beaker of fish water

egg water

Procedure

1. Place two females into the MESAB solution in each of the two 250 ml beakers. 2. When gill movement has slowed, remove one of the fish with the plastic spoon. 3. Rinse the fish in the water in the finger bowl.
4. Gently place the fish on a paper towel to dry briefly.
5. Using the spoon, transfer the fish into a small plastic dish.
6. Slightly dampen your fingers.
7. Place one finger of one hand on the dorsal side of the fish.

8. Using one finger of the other hand express the eggs by gently pressing on the ventral side of the fish, starting just behind the pectoral fins and moving toward the tail. Only gentle pressure is needed. If the fish has eggs they will come out easily. If gentle pressure fails to produce eggs do not continue to squeeze harder. Extra squeezing may injure the fish.

9. If eggs are obtained, use the metal spatula to gently move them away from the fish's body. Then slide the fish out of the dish.

10. Put the fish into a recovery container to revive.

11. When eggs are obtained by squeezing, cover them with the lid to the dish. Use eggs for fertilization within 2-3 minutes!!!

12. Repeat this for the remaining fish.

In Vitro Fertilization Procedure

1. Pipette 50 µl of Hanks/sperm onto the eggs by pushing the plunger all the way through the capillary so that the tip of the plunger extends out of the end of the capillary.

2. Gently mix the sperm and eggs with the tip of the pipettor.

3. Using the 1 ml pipette, add 0.75 ml of Fructose - egg water to the egg/sperm mixture. This activates the sperm so that they can fertilize the eggs. The time of fertilization occurs when the WATER is added, not when the sperm is added.

4. Cover the dish with its lid.

5. Allow two minutes for fertilization to complete, then add more egg water, approximately 5 ml.

Gynogenesis: Ploidy manipulation in zebrafish

Fertilization with UV inactivated sperm: Generation of haploid embryos

A. Preparation of females for squeezing

1. In each breeding trap set-up two females and one male overnight.

2. Promptly at 8:30 ( or dawn time) separate the females from the males, prior to egg laying.

B. Squeezing males for fresh sperm samples

1. Collect 1.0 µl of sperm for each clutch to be fertilized. 50.0 µl of Extension buffer(Israel) on ice, should be used as buffer for each 2.0 µl of sperm collected.

2. Samples of 5.0 µl of sperm, in 100.0 µl of Extension Buffer can be used to quicken UV treatment time(this way 25.0 µl of buffer can be used to rinse the watch glass after UV irradiation).

3. Sperm samples can be kept overnight, or collected in the morning.

C. UV inactivation of sperm

1. Fill 15 cm petri dish with ice and press clean watch glass onto surface.

2. Avoid any condensation forming on glass surface.

3. Transfer 100.0 µl sperm and buffer solution to chilled watch glass.

4. Place in Stratalinker(lid should be removed from petri dish) and irradiate for 0.3 minutes.

Note: Stratalinker should be warmed up prior to use, by running one cycle while empty. Note: if you are not familiar with the Stratalinker, test efficiency of UV light by trying to fertilize *golden* or

*albino* eggs with wild type sperm. The embryos should have the genotype and phenotype of the mother. If UV inactivates sperm, embryos should develop as nonpigmented haploids.

5. Transfer 100.0 µl UV sperm solution to fresh Eppendorf tube.

6. Rinse watch glass with 25.0 µl extension buffer resulting in 125.0 µl UV sperm solution for 5 clutches of eggs.

D. Squeezing females for egg clutch activation with irradiated sperm, immediately followed by early pressure treatment

1. Squeeze eggs into 60mm petri dish.

2. Pipette 70.0 µl UV sperm solution onto the clutch of eggs.

3. Pipette 750.0 µl fructose egg water onto the clutch of eggs,.

Early Pressure Treatment

Proceed identical to generation of haploid embryos. After fertilization: the second meiotic division is blocked by a pressure pulse>

Squeezing females for egg clutch activation with irradiated sperm, immediately followed by early pressure treatment

1. Squeeze eggs into 60mm petri dish.

2. Pipette 70.0 µl UV sperm solution onto the clutch of eggs.

3. Pipette 750.0 µl fructose egg water onto the clutch of eggs, starting the timer.

**4. Within the next 1 minute and 24 seconds:**

a. transfer as many eggs as possible to glass scintillation vial.

b. fill vial completely to form convex meniscus.

c. lay parafilm over surface, making sure no bubbles form under the film.

d. cap vial, using plastic cap with a hole punched in the top and place upside-down in pressure cell, pre-filled with water.

e. push piston into cell to second grove.

f. slide cell into press and pump to desired pressure 8000 psi. (6280 lbs for a 1 inch diameter piston)

5. When the time reaches 6 minutes turn bottom knob, which is located on the base of the press, counter clockwise to slowly release the pressure over a 1 minute time interval.

6. Transfer embryos to clean petri dish with blue egg water.

Heat shock

(C. Walker; Zebrafish book)

This method uses a heat shock to inhibit the first mitotic division of the embryo. Streisinger et al. (1981) report that 10-20% of embryos treated with heat shock develop into adults.

Overview of heat shock

1. Proceed with in vitro fertilization using UV inactivated sperm as described above.
2. At 13 min. after fertilization (at 28.5C), heat shock the embryos for 2 min. by transferring them to 41°C water.
3. Cool them rapidly to 28.5°C.

Detailed heat shock procedures

Materials Needed:

2 water baths, one set at 28.5 °C, one set at 41.4°C
3 or 4 1-liter beakers filled with egg water
Distilled water
Egg water
Heat shock vials
Electric stirrer that can be immersed
Thermometer
Timer
Data pad
Pasteur pipettes with narrow ends removed

Preparation:

1. Soak the heat shock vials over night in distilled water.
2. Fill water baths with distilled water.
3. Place one 1 liter beaker in the 41°C bath on a magnetic stirrer.
4. Fill this beaker with egg water to the level of the surrounding bath water.
5. Place 2 or 3 1 liter beakers in the 28.5°C bath and fill them with egg water to the level of the surrounding bath water.
6. Remove the heat shock vials from the distilled water and allow them to dry on paper towels.
7. Prepare data sheet. (see Example below)
8. Place a few of the heat shock vials into one of the beakers in the 28.5C bath, beginning with the vial marked '1'.

Procedure:

1. Squeeze female fish as for Embryo Production by In Vitro Fertilization and activate them with UV sperm.
2. As the 1 ml of egg water is added to begin activation, start the timer. Mark this dish #1. Each successive batch of eggs obtained should be marked in numerical order.
3. Add more egg water to the fertilized eggs after about 30 seconds.
4. About 8 minutes after fertilization, transfer embryos from their dish to the appropriately numbered heat shock vial in the 28.5°C bath. Each batch of eggs treated should be transferred to the vial marked with the same number as the number on the dish in which the eggs are fertilized.
5. At 13 min. after activation, transfer the vial containing the embryos to the 41.4°C water bath.

6. At 15 min. after fertilization transfer the vial from the 41.4°C bath to the 28.5°C bath.

7. Repeat this for the other batches of eggs. This is different from EP in that you can continue squeezing females while you are waiting to transfer the embryos from one bath to the other. Because of this, you will not restart the timer for each fertilization. For each subsequent fertilization simply record the time shown on the running timer as Time of Activation. At the time you transfer the eggs from the 28.5°C bath to the hot bath add 2 minutes to that time to find the time to transfer the eggs from the hot bath back to the 28.5°C bath. (Again, see Sample data sheet).

8. Throughout the experiment you should be monitoring the temperature of the water baths, especially the 41.4°C water bath. Do this by putting the thermometer in the beakers themselves not into the surrounding water of the baths. If the temperature is a little high you may want to add a little room temperature egg water to the beaker. If the temperature is a little low, you can remove a little water from the beaker. If the baths are holding steady at a temperature that is either too high or too low, you may need to adjust the temperature setting on the circulators of the baths.

9. When you are done squeezing fish, leave the heat shock vials in the 28.5°C bath until all the embryos have had time to divide at least once. Then remove the first vial, and transfer the embryos to beakers or dishes for sorting the fertile eggs from the infertile eggs. Record the numbers of both classes on your data sheet. Repeat this for all the other vials in the bath.

Gynogenesis: Ploidy manipulation in medaka

UV-irradiation of sperm
(K. Naruse and A. Shima)

Detailed procedures for preparing sperm suspension

Materials needed:

- Watch glass
- Pasteur pipette
- Fine forceps
- Glass needle
- Yamamoto's isotonic balanced salt solution (BSS) (Stock solution)

1. Dilute 10xBSS Stock solution with distilled water.
2. Add 1.5 to 2 ml of 1x BSS to a watch glass.
3. Decapitate 2 to 3 male fish.
4. Remove the testes gently with a fine forceps.
5. Transfer the testes to the watch glass with BSS.
6. Macerate the testes with a fine forceps and prepare sperm suspension .
7. Remove about 1 ml of sperm suspension without testicular debris with a Pasteur pipette and transfer it to a new dry watch glass. Now sperm suspension is ready for UV irradiation. Discard the pipette having been used for preparing sperm suspension to prevent cross-contamination sperm.

UV irradiation

About 1 ml of sperm suspension is put into a small watch glass and is irradiated with UV light (UV-C: germicidal lamp, predominant at 254 nm; total dose 200 J/$m^2$).

Detailed procedures for UV irradiation

Materials needed:

- Stop watch
- Glass petri dish
- Latex gloves
- UV lamp

1. Turn on UV lamp 30 min. before UV irradiation.
2. Put the sperm suspension in a large petri dish and cover with top of the petri dish.
3. Operators should put on latex gloves on his/her hands.
4. Place the covered petri dish under UV lamp where UV dosimetry was done.

5. Remove the cover of the petri dish and start the stopwatch.

6. After exposing sperm to UV for adequate time in order to make the sperm infertile (UV dose 200 $J/m^2$), replace the cover of the petri dish and remove the whole material from UV exposure apparatus. Sperm suspension is ready for use for the artificial insemination

Production of gynogenetic diploid medaka fish by early pressure treatment

(Naruse, K. and A. Shima)

Collection of the unfertilized eggs and sperm

To obtain the ripe unfertilized eggs, female medaka fish confirmed to have spawned should be separated from the males one day before the use for experiment. Sacrifice female medaka fish by decapitation and gently remove ovaries on the day of the experiment. The ovaries removed be kept in Yamamoto's isotonic balanced salt solution (BSS). The ripe unfertilized eggs be isolated with fine forceps or glass needles and be kept in a small watch glass with BSS.

Two to three males be decapitated, the abdomen be cut open. The removed testes be kept in 1.5 to 2 ml of BSS. Sperm suspension without testicular debris be prepared. In case one male is used as the sperm source, reduce the volume of BSS.

Detailed procedures for collecting the ripe eggs

Materials needed:

- Small tank
- Glass petri dish
- Watch glass
- Pasteur pipette
- Large bore size pipette
- Fine forceps
- Glass needle
- Yamamoto's isotonic balanced salt solution (BSS) (Stock solution)
- Messing cylinder

One day before the experiment

1. Females which have spawned one or more times should be isolated from males one day before the experiment.

On the day of experiment

2. Dilute 10xBSS stock solution with distilled water.
3. Add 1xBSS to a glass petri dish
4. Sacrifice female fish by decapitation and remove the ovaries with a fine forceps.
5. Transfer the ovaries to a glass petri dish with BSS.

6. Use 4 to 6 females until obtaining the desired number of eggs.

7. Separate the ripe unfertilized eggs with a glass needle or a fine forceps from ovarian tissues in BSS. The ripe unfertilized eggs can be distinguished from the unripe ones by their large size and translucency.

8. Transfer the ripe unfertilized eggs to a small watch glass with a large bore size pipette (over 1.5 mm diameter).

9. Put the small watch glass with the unfertilized eggs in a larger petri dish and cover the larger dish.

Artificial insemination and early pressure treatment

Before insemination, remove excess BSS from the watch glass containing unfertilized eggs to prevent the dilution of sperm suspension. Excess dilution of sperm concentration may decrease fertilization rate. Artificial insemination is carried out by adding the sperm suspension to the eggs in the watch glass with a Pasteur pipette.

The French press chamber should be assembled before the experiment. Eggs are put into the BSS-filled French press chamber with a large bore size pipette. The chamber is put on the French press. Within five minute after insemination ( earlier is better for good survival of embryos, but too early transfer of eggs to French press chamber may reduce the fertilization rate.), pressure is applied until the gauge reads 700 kg/cm2 and this pressure is kept for 7 minutes. Alternatively, raise the pressure to 700 kg/cm2 and keep that pressure for 6 min. and release pressure quickly and then reraise the pressure to 700 kg /cm2 and keep that pressure

for another 6 min. The latter method sometimes increase survival of embryos.

Detailed procedures for early pressure treatment

Materials needed:

Stop watch
Glass petri dish
French press
Pressure chamber
Yamamoto's isotonic balanced salt solution (BSS).
Large bore size pipette
UV irradiated sperm

1. Fill the pressure chamber with 1xBSS. (Assemble the pressure chamber before collecting the unfertilized eggs.)

2. Remove excess BSS from a watch glass with a clean Pasteur pipette to prevent dilution of sperm.

3. Add UV-irradiated sperm suspension to eggs and start stop watch.

4. Allow about 1 min. for activation.

5. Transfer the fertilized eggs using a large bore size pipette to the pressure chamber directly.

6. Assemble top cover of the pressure chamber (do not tighten the release knob).

7. Remove air from the pressure chamber through the release hole and after that, tighten the release knob.

8. Place the pressure chamber in the French press and raise the pressure to 700 kg/cm2.

9. Maintain the chamber under that pressure for 7 min. ( for total time from insemination is about 10 to 12 min.). Alternatively, leave the chamber in the press for 6 min., release the pressure and then reapply the pressure to 700 kg/cm2 for 6 min. This method sometimes increases the hatchability of the embryos. In our experience, hatchability of the treated embryos is about 20 %.

10. Remove the chamber from the press, release the knob and open the top cover.

11. Transfer the eggs from the chamber to a new petri dish with a large bore size pipette.

Keeping eggs after the pressure treatment

After releasing pressure, remove eggs from the chamber with a large bore size pipette and transfer the eggs into the fresh BSS. Keep eggs in the dark for about 2 hour to prevent photoreactivation (visible light-dependent repair of DNA damage induced in sperm by UV). Eggs are transferred to aged tap water with 0.005 % methylene blue.

Detailed procedures for keeping eggs after pressure treatment

Materials needed:

Glass petri dish

Yamamoto's isotonic balanced salt solution (BSS).

Large bore size pipette

96 well micro test plate

Tap water with 0.005% methylene blue

1. Keep eggs in the petri dish under the dark condition for about 2 hour to prevent photoreactivation.

2. Replace BSS to aged tap water with 0.005% methylene blue.

3. One day after the pressure treatment, eggs are separated from each other and inoculated in 96 well micro test plates so that individual embryos can be identified and closely observed.

Heat shock of oocytes: production of triploids and gynogenetic diploids

(D. Chourrout & C. Winkler)

1. Isolate males and females the day before heat shock

2. Dissect ripe medaka oocytes in 1x Ringer's solution at 25°C

3. *In vitro* fertilize with non-irradiated sperm (for triploids) or UV-irradiated sperm (for homozygous diploids) in 1x Ringer's at 25°C

4. After 2.5 min transfer fertilized oocytes to 41°C waterbath using a strainer

5. Incubate for 2.0 min

6. Transfer oocytes to 1x Ringer's at 25°C

7. Transfer to embryo rearing medium after 2 hrs

Induction and detection of mutations in male germ cells of the medaka by specific-locus method

(Shimada, A. and A. Shima)

Specific-locus method

The specific-locus method detects forward mutations at specific loci (marker loci) in the first-generation offspring by mating treated wildtype fish with a non-treated partner (tester) which is homozygous for the recessive alleles for these marker loci (Fig. 1). Since frequency of mutations is higher in male germ cells than in female ones in general, wildtype males are usually treated with a mutagen or mutagens, followed by mating with non-treated tester females. As the marker loci, the *b* (colorless melanophores), *lf* (leucophore free), and *gu* (guanineless) are used in the Medaka specific-locus method. The tester fish are triple recessive homozygotes at these loci.

| | | |
|---|---|---|
| P | Wildtype---------- | ----------Tester |
| +/+ | X | *a/a* |
| + -> *a** | | |
| F1 | | *a*/a* |

Fig.1 Outline of specific-locus method. "+" and "*a*" symbolize *a* wildtype and a recessive mutant allele, respectively, at the locus concerned. Mutagenized wildtype fish (+/+, in this case males) are mated with tester females (*a/a*). Any mutation (+ -> *a**) occurring at the "*a*" locus can be detected in the F1 generation on the basis of phenotype similar to that of the genotype "*a/a*".

Phenotypes of the recessive homozygotes of the marker loci

*b* locus: In the wildtype, melanophores appear on the yolk sac and embryo body on the 2nd day after fertilization. In the *b/b* embryos, melanophores are invisible although a few slightly melanized ones often appear on the surface of the head region. Use transmitting light for observation.

*lf* locus: In the wildtype embryos, yellow-colored leucophores appear beneath the brain on the 2nd day after fertilization. On the 6th day of fertilization, orange-colored leucophores appear both around the brain and on the body surface. The *lf/lf* embryos completely lack visible leucophores. Use epi-illuminating white light for observation.

*gu* locus: In the wildtype, silver to golden-colored iridocytes appear on the surface of the eye balls on the 3rd~4th day after fertilization. In the *gu/gu* embryos, the amount of guanine platelets in the iridocytes is reduced, so that the eyes and the abdomen appear black. Use epi-illumination light for observation.

Maturation stages of male germ cells

Based on the results of the study on cell population kinetics of spermatogenesis of the Medaka (Egami and Hyodo-Taguchi, 1967), maturation stages at the time of treatment are judged according to the day of fertilization after treatment as follows:

1 - 3 days after treatment; sperm

4 - 9 days after treatment; spermatids

10-15 days after treatment; spermatocytes

16-29 days after treatment; differentiating spermatogonia

30 or more days after treatment; stem spermatogonia

Procedures of the treatment of males with mutagen(s)

1. Conditioning of females and males

About 3 weeks prior to the treatment, transfer both tester and wildtype adult fish from the stock culture condition to the breeding condition. Keep each wildtype male with tester female in a plastic cage (for example, 34x17x13cm). Usually, about fifty to seventy pairs are used for one experiment. Culture the fish at 27°C under 14-hr light and 10-hr dark cycle in order to keep them lay eggs every morning throughout the year. During the conditioning period, check fertility of the males and spontaneous lethality, total mutations as well as viable mutations of the F1 embryos. To accumulate the background data is essential.

Treatment of wildtype males

Radiation treatment:

Put the wildtype males into a plastic flask containing aged tap water(10fishes /500ml water), and then irradiate them with low LET (linear energy transfer, e.g. X-rays and □-rays) ionizing radiation. The optimum irradiation dose is about 5 Gy for efficient induction of mutations. Radiation doses over 5 Gy greatly increase lethality of the F1 embryos from irradiated postmeiotic male germ cells and decrease fertility of the males after several weeks. An appropriate dose rate is about 1 Gy/min, which is generally regarded as high dose-rate. In performing irradiation experiments, to follow the radiation regulation concerned is essential.

ENU treatment:

1. Just before use, weigh ENU powder in order to get a desired concentration of ENU whose volume should be at least 20 ml more than the corresponding calculated volume. Dissolve the weighed ENU powder in the appropriate volume of distilled water by gentle but repeated pipetting with an automatic dispenser.

2. Put 2 males into a small plastic flask (capacity 25 ml) with, if necessary, the aid of a funnel, add 10 ml of ENU solution to the flask (2 fishes /10 ml). Repeat this procedure about every one minute.

3. Incubate the fish at 27°C for 2 hours in the dark.

4. At the end of incubation, separate the fish from ENU solution by powering the whole material in the flask into a strainer with a nylon mesh, rinse the fish with running tap water for several seconds in

the nylon mesh-equipped strainer and transfer the treated fish into a container with about 10 liter of aged tap water.

5. Reserve the used ENU solution and de-mutagenized the used ENU solution in accordance with the guideline of your institution. In connection with this, to minimize the volume of ENU solution might be beneficial.

6. After 1 hour, change the water.

7. After 2 hours, transfer the fish into the water containing 1~10 ppm methylene blue and 0.3% NaCl and incubate overnight in order to help recovery of the treated males from acute chemical stress.

Collection and incubation of the F1 embryos

1. Next morning, allow each of the treated males to mate with a non-treated tester female in a breeding cage.

2. Every morning collect clusters of F1 embryos from females and put the embryos into glass vessels (9cm in diameter, 6cm in depth) containing the aged tap water with about 1ppm methylene blue.

3. Two to 10 hours later, put the embryos onto the palm of your hand and separate individual embryos by rolling the cluster with your finger, thus cutting long attaching filaments.

4. Change the methylene blue solution twice in order to remove debris. This is very important because embryos can develop normally in the clean water.

5. After checking fertility, put the fertilized embryos one by one into a well of a plastic microtiter plate (96-well, U-type) with about 0.3ml of methylene blue solution, and incubate them at 27°C.

Judgment of the phenotypes of the F1 embryos

1. Observe embryos in the microtiterplates at least twice a day under a stereoscopic microscope to examine early death (ED)(death within a day after fertilization), middle death (MD)(death on the 2nd to 6th day after fertilization), late death (LD)( death after 7th day of fertilization),hatch dead(HD), or hatch viable (HV).

2. Judge the phenotypic expression of the marker loci and any morphological abnormalities such as morphology of the eye, brain, otic organs and trunk, embryo body size, angiogenesis, heart beating, and so on. Record anything you find during observation.

Calculation of mutation rates

Calculate dominant lethal (DL) rate, an indicator of lethality of the whole embryos, total mutation (TM) rates which include mutations associated with dominant lethals, and viable mutation (VM) rates which correspond to the "mutation rate" generally used in animal experiments.

Spontaneous lethal rate=(no. of fertilized eggs - no. of viable fry )/(no. of fertilized eggs)

DL rate=(observed lethal rate - spontaneous lethal rate)/(1 - spontaneous lethal rate) (by Abbott's correction formula)

TM rate=(no. of mutations)/(sum of the effective no. of loci per embryo*)

VM rate=(no. of viable mutations)/(no. of viable fry x 3)

*Effective no. of loci per embryo

| Morphological abnormalities of embryos | Effective no. of loci per embryo | | | |
|---|---|---|---|---|
| | b f | l u | g | t otal |
| Embryo without embryo body or without distinct head or tail | 0 | 0 | 0 | 0 |
| Embryo with distinct head or tail but embryo body encircles less than 1/2 of yolk sac | 1 | 1 | 0 | 2 |
| Embryo whose embryo body encircles more than 1/2 of yolk sac | 1 | 1 | 1 | 3 |

References:


Mullins, M.C., M. Hammerschmidt, P. Haffter & C. Nusslein-Volhard (1994) Curr. Biol., 4:189-202.

Shima, A. & A. Shimada (1988) Mutation Res., 198: 93-98.

Shima, A. & A. Shimada (1991) Proc. Natl. Acad. Sci. USA, 88:2545-2549.

Shima, A. & A. Shimada (1994) Environ. Health Perspect., 102:33-35.

Solnica-Krezel, L., A.F. Schier & W. Driever (1994) Genetics, 136:1401-1420.

Blastomere Lineage Analysis

Note: The protocol below uses injection of fluorescent dye into single cells for labeling. Recently, the use of caged fluoresceins has become popular: embryos are dye filled, and fluorescent dyes locally released by illumination with laser light or the microscope UV epiilumination used for normal fluorescence microscopy. We will likely show some use of caged fluoresceins at the course.

An excellent source for updated information on fluorescent dyes is the Molecular Probes Web site : www.probes.com

There is also an image of a zebrafish embryo from an uncaging experiment (http://www.probes.com/cgi-bin/photo.cgi?region=Select+Region&file=g000496

by Walter Metcalfe)

(Source: R. Warga and C. Kimmel)

This protocol provides methods for labeling blastomeres with tracer dyes and observing their clonal progeny in live or fixed whole-mounted embryos. An alternative method available today is the use of caged fluoresceins: the embryo is dye filled early with the caged fluorescent dye, but only cells which have been illuminated at a defined wave length will have fluoresceine "released from its chemical cage".

Dissolve the dyes in 0.2 M KCl (at the concentrations specified below) and centrifuge through a 0.20 µm pore filter before filling the pipettes.

Tetramethyl-rhodamine Dextran (10,000MW, neutral: Molecular Probes) make a 5% solution. This is the standard dye used for fate mapping and lineage tracing.

Fluoresceine-isothiocyanate Dextran (10,000MW, neutral: Sigma or Molecular Probes) make a 5% solution. This dye is not particularly good for repeated observations. It fades quickly, and causes substantial photo damage to the cells during long exposures to fluorescent light. Another disadvantage is that the embryo itself, particularly the yolk, has considerable autofluorescence at the wavelengths used to view fluoresceine.

BiotinDextran (10,000MW, lysine fixable: Molecular Probes) make a 3% solution. This dye is used as a fixable tracer, and can be included in the injection pipette along with the rhodamine dextran if permanent preparations are desired.

Dye-filling the microinjection pipettes:

Make micropipettes from thin-walled Borosillicate capillary tubing, with the rapid fill glass fiber (e.g. Frederic Hear Inc. or Sutter Glass). Pull a rather steep taper and a very sharp tip on the injection pipettes with a standard puller (e.g. a Flaming Brown programmable horizontal micropipette puller). When determining the settings, check the point under a compound microscope at about 400x; it should be so sharp that the taper seems to disappear to nothing. Later this tip will be broken off, and the taper largely will determine what the size of the tip will be after breaking it. Backfill the injection pipette tips by placing a drop of the dye solution on their butt ends. The fiber inside the pipette allows the dye to run up to the tips, where it accumulates. Generally bubbles are present, but many will gradually

disappear. Hence, the pipettes can be filled the day before use, and stored in a refrigerator in a humidified container.

The injection rig:

Standard methods borrowed from electrophysiologists are used to label cells. The basic setup includes a stimulator, an intracellular amplifier, and an oscilloscope, and you can learn how to hook the boxes together from a cooperative physiologist if you don't know how to do these things. An audio monitor for hearing the voltage is also very useful. The holder for the injection pipette is mounted on a micromanipulator (e.g. a Leitz manipulator, which gives lovely control and stability, and a hydraulic advance fitted in front of it, permitting very fine control of axial excursions of the pipette). For blastomere injections, a relatively inexpensive Nomarski microscope with 10x and 20x objectives can be used. Even though the lenses are corrected for a coverslip, the optics are adequate without. The working distance for either objective is sufficient to position the injection pipette underneath, if you mount it with the manipulator so that the pipette comes in at a shallow angle, about 20° from the horizontal, just clearing the microscope stage. To position the pipette near the cell of interest use both the manipulator and microscope stage controls.

The holder for the injection pipette (WPI, Inc.) permits recording, passing current, and dye injection by pressure. The pressure can be regulated with a commercial unit (e.g. General Valve, Corp.) or with a simple system built from individual parts. It is important to regulate the pressure both up and down agreeably and without hysteresis, while the pipette tip is inside the cell.

The "air in" in this system can come from an in house supply of air, but a nitrogen or compressed air tank can provide higher pressures. The "air out" goes directly to the pipette. Just before use, backfill the pipette (i.e. from its butt end) with 0.2 M KCl applied behind the dye solution. You can buy a long thin syringe needle for this purpose (Hamilton) or pull out a suitably long capillary from a Pasteur pipette. Mount the pipette on the holder. With another manipulator, advance a glass rod firepolished at the end to produce a ball about 3 mm in diameter. This ball is used to break the point of the pipette by gently advancing it against the ball, under the 20x objective. After breaking you will be able to see that the end is just a bit blunt. A good pipette for injection, even in the small blastomeres of a late blastula, will have a resistance of about 80 megohms, as measured in a bath of 30% Danieau's. The bath is grounded by an agar-bridged silver-silver chloride ground wire.

Agar bridged ground wires

Plate silver wires electrolytically with chloride or dip into molten silver chloride. (Dipped wires last longer than plated wires.) Insert the silver chloride ground wire into a 15 cm length, small diameter PE tube filled with 3% agar in 30% Danieau's; this is the agar bridge. The ground wire should insert only about halfway into the agar. Seal the agar bridge to the ground wire with epoxy and heat shrink tubing. Store in the refrigerator with the tubing immersed in 30% Danieau's.

Preparing the Embryos

About an hour after fertilization, stage, and clean. Under a stereo dissection microscope, remove the chorions with fine sharpened watchmakers forceps (Dumont no. 5) by gently teasing open a hole in the chorion, widening it sufficiently and carefully shaking out the embryo (see Removing Embryos from their Chorions, Chapter 4, for more details). This is done in 30% Danieau's, in agar-coated petri dishes (2% Difco Bactoagar). Once the embryos are free of the chorion, transfer with a fire polished pipette through several rinses in 30% Danieau's to a clean agar dish. Using both 30% Danieau's and agar dishes greatly improves survival during and following dechorionation. 30% Danieau's contains elevated Ca2+ which helps to keep the cells intact, and the agar provides a smooth nonsticky surface for the embryos to sit. If

you put a dechorionated embryo at cleavage or blastula stage directly on the plastic surface of a dish, and carry the dish around, you may end up with fragments or a dissociated suspension of dead cells. After the injection, return the embryos to agar dishes for incubation, unless they are to be viewed immediately. For the injection, mount the embryo in a depression slide in a drop of 30% Danieau's, or if you need to orient it in a position in which it won't lie still, use 3% methyl cellulose in 30% Danieau's (see Methyl Cellulose Mounting).

Labeling the embryos

Use current injection to drive the electrode through the cell membrane. Blastomeres take an unusually high amount of negative current, driven by as much as 14 V with a duration of about 50 msec. This method is vastly preferable to manually advancing the tip through the membrane or wiggling it through by capacitance "ringing". Under visual control (20 x objective) advance the electrode against the cell of interest until you can see it dimple the cell surface. Then give it a blast of current and it should penetrate. You often see the dimple disappear, and if you are monitoring voltage through the injection pipette you will see a negative 30 mV or so (as high as -70 mV) resting potential. If you have damaged the cell, or if the electrode has passed completely through the cell into the small amount of extracellular space within the blastoderm, you will observe a positive change, not a negative one. Deep cells can be penetrated by very prominently dimpling an EVL cell that has a deep cell positioned just beneath it. After the current injection, the pipette will have usually advanced through the thin EVL cell and into the underlying deep one. Expel dye through the pipette by applying positive pressure, to lightly color the cell. You can monitor the dye injection on a rig that is not equipped with fluorescence optics simply by watching the amount of color in the blastomere under Nomarski. The dye should diffuse throughout the whole cell, not brightly label a small dot-like inclusion within the cell. If you can see the color easily with your eyes, you may have filled the cell too brightly. You should not notice a volume change in the cell nor any evidence of damage (e.g. leaking cytoplasm) as you withdraw the pipette from the cell. Generally you need fill the cell for only a few seconds. If you are doing a clonal analysis and want to be sure that only a single cell is labeled, you need to look at the embryo immediately after the injection with fluorescence optics and image intensification. There is no other way to be certain that dye has not spuriously labeled neighboring cells.

Single looks at the clone

For a short-duration look at the labeled cells, as you might do in a fate mapping experiment, mount embryos in 3% methyl cellulose on a glass depression slide by gently inserting the embryo into a drop of methyl cellulose or by laying it on top of the gel, depending on how much methyl cellulose you wish to use. Carefully orient the embryo using a nylon hair loop. Add a thin film of 30% Danieau's to cover the drop of methyl cellulose, and view it with no coverslip. (See Methyl Cellulose Mounting, for more details.) Screen the clones with the aid of an image intensifying camera (e.g. Videoscope) mounted on a compound microscope. With signal intensification, one can detect very lightly labeled cells, using very low levels of exciting light, thereby avoiding photodamage to the labeled cells. This is particularly important if you are going to view them repeatedly. The camera output is sent directly to a video display monitor and also can be stored on a variety of storage devices, (e.g. a computer hard disk, an optical disk, or a VCR).

Labeling Single Cells With Lineage Tracers

(Source: B. Melby and D. Raible)

This is a technique for intracellular labeling of small cells at gastrula stages or later.

Electronics

The electronics set-up is used as a means to get the dye out of the electrode and into the cell. Use an agar bridge (see Agar bridged ground wires) for the bath ground.

Pipettes

Success in single cell labeling lies primarily with the ability to make good pipettes. Pipettes should be pulled the day of the experiment. Allow at least 10 min for the tips to fill with dye. Electrodes are pulled from glass capillaries (e.g. Sutter thick wall, O.D. 1.2 mm, I.D. .69 mm) with an inner filament for easy dye filling.

The ideal pipette tip can be attained only by fine-tuning and testing. The pipettes must be sufficiently sharp to pierce through the outer layer of the embryo, yet have a large enough tip diameter to pass dye. They should have a long, sharp shank but a blunt tip. An important feature of the pipettes is that they pass dye, either by current injection or by "ringing" the capacitance, and that they do not continuously leak dye. The resistance of the pipettes provides a measure of reproducibility from one experiment to another. Good pipettes usually have a resistance in the range of 100-300 Mohm when filled with 0.2 M KCl. If the resistance is too high, it may indicate a problem with the dye, or with the bath ground, as well as with the pipette shape.

The dye should be dissolved in 0.2 M KCl and filtered (e.g. 0.2 µm spin filters for the microcentrifuge). A 3% solution of rhodamine dextran (Molecular Probes) works quite well. Dye can be made in advance and stored at 4°C in a sealed tube. To fill pipette tips with dye, place a droplet of dye (about 0.5 µl) at the back of the pipette.

Labeling gastrula stage cells

Embryo preparation and mounting:

Embryos should be removed from their chorions in agar-coated dishes using fine forceps. After removing the chorions, transfer the embryos with a fire-polished pipette. Experimental slides are made by painting several coats of nail polish in a ring of about 3 cm diameter, on a glass slide. Mount embryos in methyl cellulose (see Methyl Cellulose Mounting) to permit easy manipulation and reorientation. Chilled methyl cellulose is stiffer, but tends to get bubbly as it warms up, therefore it is best to use methyl cellulose at room temperature.

1. Spread a layer of methyl cellulose in the center of the experimental slide.
2. Cover with 30% Danieau's, being careful not to spill over the nail polish ring.
3. Transfer an embryo to the center of the slide. Orient the embryo under the dissecting microscope by gently nudging it with a hair loop.
4. When the embryo is in the preferred orientation, gently tamp it down into the methyl cellulose. Over time, the methyl cellulose will become diluted with 30% Danieau's and will become too fluid, so that the embryo may roll. If this happens, remove the embryo and remount.
5. Be sure to loosen the embryo from the methyl cellulose before trying to move it with a pipette.

Labeling cells:

It is easiest to label cells if the embryo is oriented such that the surface to be labeled is at the top, lying flat. The ideal situation is to have the embryo oriented so that the cells to be labeled are easily visible in reference to landmarks such as the embryonic shield or the margin.

1. Check the orientation of the embryo at low and then high magnification, to be sure that it is appropriate.
2. Place a hanging droplet of 30% Danieau's on the water immersion lens (e.g. 40x) and gently slide the lens into place over the embryo, to avoid rolling the embryo.
3. Position the bath ground at the back of the slide, being sure that there is continuity between the agar and the 30% Danieau's.
4. Raise the microscope objective so that there is plenty of space between the objective and the embryo.
5. Backfill a pipette with 0.5 M KCl, place it in the pipette holder, and attach the holder to the amplifier probe which should be mounted on the micromanipulator. There should be no large bubbles in the pipette or holder; they will cause electrical discontinuity.
6. Advance the pipette into the 30% Danieau's and position it above the embryo using the white light and by viewing it from the side of the microscope.
7. Locate the tip under magnification, and check to make sure that it isn't broken.
8. Switch on the amplifier, this should only be turned on when there is a complete circuit (i.e. from probe, through the bath and bath ground, back to the amplifier).
9. A good pipette will release a small puff of dye when the capacity compensation (usually a button, sometimes a dial) is turned on (i.e. "ringing the capacitance"), or when the pipette check is pressed (this injects a small amount of current). A bad pipette will leak, or sometimes not release dye at all. Throw it away and get a new one, but be sure to turn off the amplifier before you pull the pipette out of the bath.
10. Carefully lower the pipette to the level of the embryo, keeping it in focus. Superficial cells can be labeled by positioning the pipette above the cell and lowering the tip through the EVL layer until the cell membrane dimples.
11. Monitor the fluorescence, and label the cell by ringing the capacitance, or by injecting current. Ringing the capacitance causes the pipette tip to vibrate, enabling penetration and it will also cause a little bit of dye to be released.
12. After a cell has been labeled, rapidly remove the pipette using the stage control or the micromanipulator, and turn off the amplifier.
13 To label cells in deeper layers, it is best to bring the pipette in from the side. Focus on the cell layer of interest. Bring the pipette into the same focal plane as this cell layer. Advance the pipette up to the cell using a microdrive, or fine micromanipulator adjustment.

Sometimes cells along the pipette track tend to be labeled by dye leaking from the tip. The tip may appear to have passed through these cells, but there is still continuity with their membranes. Single cell labels can be achieved by stopping dye injection as soon as one cell starts to label. Because of this problem, it is important to check the fluorescence without Nomarski optics (it will be much brighter) in order to see all of the cells that have been labeled.

Localizing the labeled cell:

For mapping purposes, it is best to check the cell's location using three different orientations of the embryo. In the orientation in which the embryo was labeled, check the cell's distance from the margin

under the 40x water immersion lens. Then turn the embryo onto it's side and check the cell's radial depth at 40x. It is important to have a fairly precise side orientation, or else the cell may appear deeper or shallower than it is. The depth can also be assessed by focusing up and down in the original orientation, although this method gives no information about the relationship of the cell to the hypoblast. Finally, assess the position with respect to the dorsal midline by orienting the embryo animal pole up and looking at it under low power (10x objective).

Labeling single cells in older embryos

1. Mount embryos in agar as described in the section on Agar Mounting (Chapter 4). Orient the embryo so that the cell of interest is visible with Nomarski optics and accessible with the pipette.

2. Focus on the cell of interest, and bring the pipette along side the embryo in the same focal plane.

3. Using the stage controls, force the mounted embryo against the pipette until the skin springs back and the pipette tip enters the embryo.

4. Move the embryo away with the stage controls until there is no compression of the embryo by the pipette.

5. Then, using the fine control of the micromanipulator, move the pipette into position against the cell to be labeled. For cells close to the surface, the pipette can be inserted into the embryo at a different focal plane.

6. Label the cell as described above, and quickly remove the pipette from the embryo by moving the embryo away with the stage controls.

Preparation of chromosomes

**(D. Chourrout, C. Winkler & Y. Hong)**

1. Incubate 2 day old embryos in embryo rearing medium supplemented with 0.2 g/l colchicine (Serva) for 2 hrs at 28°C
2. Transfer embryos to 1x Ringer's; remove chorion
3. Incubate embryos in trisodiumcitrate (8 g/l) for 20 min
4. Fix in freshly prepared ethanol (-20°C)/acetic acid 3:1 (v/v) for 30 min (to o/n)
5. Change fixative; fix again for 15 min
6. Transfer single embryos to microwell with 20 µl 50% acetic acid
7. Incubate for 2 min
8. Spot dissociated embryos onto a slide on heating plate at 50°C
9. After 30 sec remove excess liquid; spot liquid again
10. Repeat twice
11. Air dry slides
12. Stain in freshly prepared 4% Giemsa solution in PBS (pH 6,8) for 30 min
13. Wash twice in water
14. Air dry slide

HISTOLOGICAL METHODS

Lac Z stain of zebrafish

(Anders Molven)

1. Fix embryos at 4°C for 30 minutes in:

| | | |
|---|---|---|
| 2% paraformaldehyde | for 5 ml: | 1.25 ml of 8% soln. |
| 0.2% glutaraldehyde | | 125 µl of 8% soln. |
| 0.02 % NP-40 | | 10 µl of 10% soln. |
| 1 x PBS | | 1 ml of 5x soln. |
| | | add 2.62 ml water to 5 ml |

2. Rinse 3 times in 1 x PBS (2-3 min each)
3. Stain (15 min to 24 hrs) at 30°C
4. Mount in Glycerol

Staining solution:

| | | | final conc. |
|---|---|---|---|
| A | 0.2 M Na phosphate buffer pH 7.3 | | 50µl (10 mM) |
| | 5 M NaCl | 30 µl | (150 mM) |
| | 1 M $MgCl_2$ | | 1 µl (1mM) |
| | 0.1 M $K_4$ ($Fe_3$ $(CN)_6$ ) | | 30 µl (3.1 mM) |
| | 0.1 M $K_3$ ($Fe_2$ $(CN)_6$ ) | | 30 µl (3.1 mM) |
| | dd water | | 859 µl |

(make stock and store as frozen aliquots)

B 8% X-gal (5-bromo-4chloro-indolyl-ß-D-galactoside) in DMF
(store frozen aliquots)

Mix 974 µl A and 26 µl B immediately before use (a precipitate may form upon mixing, does not disturb staining reaction)

10.2 OCT embedding for cryostat sectioning of embryos or larvae
(R. Bremiller)

The compositions of the solutions are listed in the "Recipes" section.

1. Fix animals as desired.
2. Wash in fix buffer, 3 times for 5 min each wash.
3. Soak fixed fish in 30% sucrose until they sink.
4. Transfer fish to embedding chamber (plastic test tube stoppers or inverted Beem capsules whose tips have been cut off) filled with OCT cryostat embedding medium (Tissue Tek).
5. Freeze chamber with a blast of compressed CO2 or by dipping chamber slowly and gradually into liquid N2.
6. Frozen blocks can be stored at -70°C if they are wrapped well so that the tissue does not dehydrate.
7. Cut 10-20 µm sections on a cryostat at -20°C, transfer them to subbed slides and let them dry completely.
8. Sections can be stored at -20°C in a sealed slide box.

### 10.3. JB 4 plastic methacrylate section

(W. Driever)

In many cases one wants to take a detailed look at in situ hybridization embryos or labeled clones from transplantation experiments. We found that semi-thin section using methacrylate type resins are easy to perform and give excellent results. You need microtome for semi thin sections, equipped for use of glass knifes for this procedure.

Materials

All solutions needed for embedding embryos are found in the JB-4 Plus Embedding Kit from Polysciences, Inc., order no. 18570. In addition, you will need EBH-2 block holders, order no. 15899, and molding cup trays with dimensions 6W x 8L x 5H mm, order no. 17177A, both available from Polysciences.

Preparation of JB-4 Catalyzed Infiltration Resin

The JB-4 Embedding Kit includes instructions for preparing the infiltration resin:

1. In the designated, foil-wrapped 200 ml bottle, combine 0.90 grams of dry catalyst C to 100 ml of JB-4 Solution A (stored in refrigerator).
2. Mix until dissolved (circa 30 minutes).
3. Store for up to 4 weeks at 4˚C.

For preparation of the methylene blue II stain see protocol from Humphrey and Pittman (1974).

Preparing Embryos

Fixation

1. Dechorionate embryos.
2. Transfer embryos to labeled Eppendorf Tubes.
3. Take off egg water and add 4% formaldehyde in PBS.
4. Lay tubes on their sides to expose all embryos to the fixative equally well.
5. Allow embryos to stand in solution 4 hours at room temperature or overnight at 4˚C.
6. Wash with PBS

Agarose Blocks

If perfect longitudinal sections are desired, after fixation single embryos can be first embedded in Agarose blocks to insure proper orientation.

1. Prepare a solution of 1% Agarose in PBS.
2. Microwave solution on high for two minutes or until Agarose is completely dissolved.
3. Place solution in 56˚C water bath to inhibit solidification.
4. Transfer fixed embryo to mold (Polysciences, order no. 17177A).
5. Fill mold with Agarose medium.
6. Under a microscope, orient embryo with a needle. The fish should lay flat on the bottom of the mold and on one side. Only one eye should be visible. The orientation should be done at the time the agarose solidifies - otherwise it moves again and lays down on its "side" - which is usually at 60 degree ventral lateral angel or so.
7. After medium has solidified, remove block from mold.

8. With a razor blade, trim block evenly into a rectangular cube. One wall should be parallel to the anterior posterior axis of the embryo.

Proceed with dehydration, infiltration, and embedding as indicated in protocol.

Dehydration

1. Dehydrate embryos using the following ethanol concentrations:

50%, 70%, 85%, 95%, twice 100%

Wait 10-15 minutes between each washing.

Embryos can be stored for several days in 70% ethanol, if necessary.

Infiltration

1. Transfer embryos in 100% EtOH to labeled 5 ml narrow, glass scintillation vials.

2. Remove liquid and add JB-4 infiltration resin, filling the vial halfway (use glass pipet or one way plastic). The liquid creates a column and allows the embryos to sink to the bottom as infiltration proceeds.

3. Place vials overnight at 4˚C.

Embedding Embryos

Always wear gloves and work under a hood with microscope, if possible.

1. Prepare an ice tub to use for keeping vials and solutions cold.

2. Decide how many blocks are desired and label the corresponding block holders with pencil to identify each block.

3. Mix the following amounts of solutions in a 15 ml tube according to the amount of blocks desired:

| JB-4 Infiltration Resin | JB-4 Hardner (B) | Number of Blocks |
|---|---|---|
| 6.25 ml | 0.25 ml | 4 |
| 12.5 ml | 0.5 ml | 8 |
| 25 ml | 1.0 ml | 16 |

4. Pipet up and down until thoroughly mixed, keeping solution on ice while mixing.

5. Pipet off JB-4 infiltration resin from embryos in scintillation vials and discard into waste collection vial. For agarose blocks: add the block first and then the embedding solution.

6. Pipet on embedding solution.

7. Transfer embryos and liquid to molds. Make sure the mold disc is filled halfway. If mold contains too much or too little liquid, adjust appropriately.

8. Under a microscope, place embryos in the center depression of the mold, allowing them to sink to the bottom. Center embryos in the middle of the mold, leaving equal room on both sides for block trimming and room on one end for picking up sections while cutting.

9. Orient embryos with a needle.

If **longitudinal sections** are desired, place embryos next to each other on their sides, point all heads in one direction, and allow to lay flat on the bottom of the mold. Agarose blocks can also be used to insure proper orientation (see Agarose protocol).

If **cross-sections** are desired, place embryos flat against the bottom of the mold. Wait a few minutes for the embedding medium to begin polymerization. Then, lift the tail of the embryo with a forceps, standing it on its head. The medium will hold it in a vertical position as polymerization starts. This can take up to ten minutes or so but test the medium often, continually trying to stand the embryo on its head. Do not wait too long to orient fish. Timing is critical: **you got only 5 - 10 minutes!**.

10. Once embryos are oriented and medium begins polymerization, securing embryos in their place, cover molds with labeled block holders.

11. Allow blocks to harden at room temperature for one hour or overnight.

12. When ready, pop blocks out of mold to section.

Sectioning Embryos

Making Knives

For any specific questions concerning the Knifemaker II from Leica, consult the operating instructions manual.

Always make glass knives as you need them because they become dull if stored too long.

Never touch knife to any surface. This immediately ruins the knife's sharpness.

1. Using the knifemaker, score and break glass into square blocks. Each block yields two knives (opposite corners). You should see a "right-hand" curve in the knives to be used.

2. Inspect each knife for faults and choose only good knives for sectioning.

3. Knives need replacing when sections become "milky" or start to roll up tightly.

Sectioning

1. Turn on heating unit for drying sections and prepare a water bath using distilled water and a few drops of ammonia. This water bath is used for straightening sections before they are mounted.

2. Select thickness of sections by turning dial. Usually, sections are cut 5 microns thick.

3. Trim block on two sides with a razor blade, eliminating excess plastic from sections. Block surface should look like a rectangle.

4. Mount block by clamping it into place. Orient block-face lengthwise.

5. Move block back completely to allow for adjustment by pressing the double-arrow key. Light will flash when block is completely back. Press the single-arrow key to move block slightly forward until the blinking light goes off.

6. Put knife in holder and secure. Make sure knife is straight and flat against holder.

7. Move knife toward block until the two almost touch.

8. Loosen pegs and adjust knife and/or block so that knife edge is straight with block. This is the critical step in obtaining good sections.

9. Once knife is properly oriented, select cutting setting of 1, 2, or 3. Setting 3 is used for the actual sectioning as it stops after each section, allowing you to collect them one by one. Setting 2 is only used to trim the block if the fish is not flat against the surface. On this setting, the machine continually cuts until "run/ stop" is pressed. In addition, you can manually cut sections but this is not as efficient for obtaining many sections.

10. Set cutting speed to around 8.

11. After selecting setting (usually 3), press "run/stop" button to cut.

12. Using two sets of dry forceps, carefully remove section from knife without touching the knife edge. Pick up section on end where there is no trace of embryo to avoid ruining section.

13. Drop section into water bath, allowing it to straighten. It is important that the forceps are dry as the section will easily stick to them if wet.

14. Take a pre-labeled slide and place it in the water bath under the section. With dry forceps perpendicular to section surface, gently move section until it floats above slide. Always touch section on its surface with dry forceps or else it will stick to the forceps and is ruined.

(alternatively, sections can be stretched on drops of water positioned on a microscope slide on a heating block. Some people bring a cotton tab with chloroform close to the water drop before placing the section on top of it. I supposed to help stretching of the section.

15. Once section is properly oriented in the water bath, slowly raise slide so that section rests upon it.

16. Place slide with section on heating unit to dry. Once all water disappears from section, it cannot be washed off in the water bath.

17. Proceed with cutting and collecting sections, aligning them side by side and in two rows on slide. Allow sections to dry completely before staining.

If knife needs to be replaced or adjusted, always realign block before cutting. Also, if you interrupt your sectioning, upon returning, always realign the knife and block before proceeding because the block continues to expand.

Note: You can recycle the block holders by pulling the resin block off the holder with two pairs of pliers.

Staining Sections

1. Place slides in a plastic slide holder.

2. Microwave about 70 ml of methylene blue (see Humphrey & Pittman protocol) for 2 minutes on medium heat.

3. Pour stain over slides and wait 1 minute.

4. Pour off stain (collect to be reused) and rinse well with distilled water.

5. Air dry or place on heating unit until dry.

6. Using Permount, place cover slip over sections and allow to dry completely.

Whole-mount *in situ* hybridization in zebrafish

Ch. Thisse and B. Thisse

Probe :

Work has to be done using sterile tubes and buffers and gloves

- <u>DNA preparation</u> :

5mg of DNA is linearized with the appropriate restriction enzyme during 2h. The reaction is then stopped using first a mix of phenol/chloroform, then chloroform. The DNA is then Ethanol precipitated, centrifuged and washed with RNAse free 70% Ethanol. DNA is then resuspended in Tris EDTA 10mM/1mM and an aliquot is tested on agarose gel.

- RNA probe synthesis :

Transcription mix :

| | |
|---|---|
| 1mg linearized DNA | |
| Transcription buffer (T3 ou T7 RNA polymerase) : | 4µl |
| NTP-DIG-RNA (Roche) : | 2µl |
| RNAse inhibitor (35u/µl) : | 1µl |
| T3/T7 RNA polymerase (20u/µl, Stratagene) : | 1µl |
| Sterile water : | up to 20µl |

- 2h at 37°C
- Digest the template DNA by adding 2µl RNAse free DNAse during 15min at 37°C.
- Stop the synthesis reaction and precipitate the RNA with 1µl EDTA 0.5M pH 8, 2.5µl LiCl 4M and 75µl cold Ethanol 100%. After storage at-70°C during 30min, centrifuge at 4°C, during 30min at 12OOO rpm, wash with 70% Ethanol, dry and resuspend in 20µl sterile DEPC water.

Test 1µl on agarose gel. (generaly 1µl will be used for the hybridization).

In situ hybridization

- Fix embryos in 4% paraformaldehyde (PFA) in PBS overnight at 4°C.
- Remove chorions manually.
- transfer embryos in 100% Methanol (MeOH), store them at -20°C (2h-several months).

In situ Day 1 :

- Rehydratation :

put embryos is small baskets and rehydrate by successive incubtions in :

5min in 75%MeOH-25%PBS
5min in 50%MeOH-50%PBS
5min in 25%MeOH-75%PBS
4 x 5min in 100% PBT (PBS/tween20 0.1%)

- Digestion with Proteinase K (10µg/ml)

Blastula and gastrula : Ø, early somitogenesis : 1min; late somitogenesis (14 to 22 somites) : 4min, 24h : 10min, 36h/48h embryos : 20min.

(One batch of embryos is used for the antibody preabsorption.

- Refixation 20min in 4%PFA-PBS
- Washes 5 x 5min in PBT
- Preadsorbtion of the anti-DIG antibody (Roche) in a 1000 dilution in PBT-sheep serum 2%-BSA 2mg/ml for several hours at room temperature with the batch of embryos previously prepared.

Prehybridization and hybridization :

tRNA and Heparin are added for prehybridation and hybridization only. (% of formamide varies function of the stringency).

Hybridization mix :

| | |
|---|---|
| - Formamide | 50 - 65% |
| - SSC | 5 x SSC |
| - Heparin | 50 µg/ml |
| - tRNA | 500 µg/ml |
| - tween 20 | 0.1% |
| - citric acid | to pH 6.0 (460 µl of 1M for 100 ml) |

Prehybridization :

2 to 5 hrs at 70°C in 800 µl of hybridization mix.

Hybridization :

Prehybridization mix is removed, discarded and replaced by 200 µl of hybridization mix containing 100 - 200 ng of antisens RNA probe. Hybridization is performed overnight, in a waterbath at 70°C.

Day 2 :

Washes : .

Embryos are transfered into small baskets fixed in floaters. Subsequent washes and incubation are carried out by letting the floater swim under agitation in 200 ml of the adequate solution.

- 1 x fast in 100 HM at 70°C.
- 15 min in 75% HM/25% 2 x SSC at 70°C
- 15 min in 50% HM/50% 2 x SSC at 70°C
- 15 min in 25% HM/75% 2 x SSC at 70°C
- 15 min in 2 x SSC at 70°C
- 2 x 30 min in 0.2 x SSC (for normal stringency) or 0.05 x SSC (for high stringency).
- 10 min in 75% 0.2 (0.05) x SSC/25% PBT at room temperature (RT)
- 10 min in 50% 0.2 (0.05) x SSC/50% PBT at RT
- 10 min in 25% 0.2 (0.05) x SSC/75% PBT at RT
- 10 min in PBT at RT
- several hours in PBT/2% sheep serum/2mg:ml BSA at RT

Incubation with anti-DIG antiserum :

- Preadsorbed anti-DIG is used at the 5000 x dilution in PBT/2% sheep serum/2mg:ml BSA overnight with agitation at +4°C.

Antiserum is preadsorbed on the first day against

Day 3 :

Washes :

Antiserum is removed and discarded. Embryos are then extensively washed :

- 1 x fast in PBT at RT
- 6 x 15 min in PBT at RT
- 3 x 5 min in solution buffer (100 mM tris HCl pH9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% tween 20)

Staining :

staining is performed at RT and monitored with a dissecting scope. Staining solution :

- 225 µl NBT 50 mg/ml
- 175 µl BCIP 50 mg/ml

- 50 ml staining buffer

(NBT stock : 50 mg Nitro Blue Tetrazolium in 0.7 ml of Dimethylformamide anhydre + 0.3 ml $H_2O$. BCIP stock 50 mg of 5-Bromo 4-Chloro3Indolyl Phosphate in 1 ml Dimethylformamide anhydre).

Reaction is stopped by removing the staining solution and washing embryos in stop solution : PBS pH5.5, EDTA 1mM. Embryos are then store in stop solution at +4°C in the dark.

Mounting :

- embryos can be observed directly in stop solution at the dissecting scope
- embryos can be mounted directly in 100% glycerol for observation at the compound microscope.
- Embryos at early developement stage (up to 18 hrs) are first dehydrated in 100% Methanol, then clarified few minutes in methylsalycilate and then mounted in Permount.

---

Materiels and supplies :

- paraformaldehyde (*Sigma*)
- 10 x PBS
- Methanol
- Tween 20 (*Sigma* P1379)
- Proteinase K (*Roche* 1000144)
- Anti DIG antibody - alkaline phosphatase Fab fragment (*Roche* 1 093 274)
- BSA fraction V protease free (*Sigma* A-3294)
- desionized Formamide (high purity grade)
- 20 x SSC
- Heparin at 5 mg/ml (*Sigma* H3393)
- RNAse free tRNA (50 mg/ml) (*Sigma* R7876, resuspended in Water and extensively deproteinized by series of Phenol/CHCl3 extractions)
- citric acid 1M
- Normal Sheep serum (*Jackson immunresearch* 013 000 121)
- Tris HCl pH9.5 1M
- MgCl2 1M
- NaCl 5M
- NBT 50 mg/ml (made from powder. *Sigma* N6876)
- BCIP 50 mg/ml (made from powder. *Sigma* B8503)
- PBS pH5.5
- EDTA 0.5M
- Glycerol 100%
- Methylsalycilate (Sigma M6752)
- Permount (*Fisher* SP15-100)

- Waterbath at 70°C (with shaking).

- orbital shaker. (horizontal shaker).

---

# RECIPES

**Zebrafish**

Agar/Sucrose:

1.5% agarose

5% sucrose Boil into solution and store as 1.5 ml aliquots at 4°C

BT Fix

4.0 ml 10% paraformaldehyde

5.0 ml 2x Fix buffer

Adjust pH to 7.3 if necessary. Add $dH_2O$ to a final volume of 10 ml. Final concentration is 4% paraformaldehyde, 0.15 mM $CaCl_2$, 4%sucrose in 0.1 M $PO_4$ buffer

Chorion Removal

Drain eggs in a 5 cm Petri dish

Add 5 ml of 0.5 mg/ml pronase until the first embryos pop out of the chrion (for about 3.5 min)

Dilute eggs with 200 ml of Egg Water. Rinse 3x more with 200 ml washes of Egg Water and transfer soon to 30% Danieau's.

Danieau's solution for incubation of dechorionated embryos

Full Strength:

| | | Stock soln. | | use per L |
|---|---|---|---|---|
| 58 | mM NaCl (MW= 58.44 g/mol) | 50x | 169.5 g/L | 20 ml |
| 0.7 | mMKCl (MW= 74.44 g/mol) | 100x | 5.2 g/L | 10 ml |
| 0.4 | mM$MgSO_4$ x 7 $H_2$ O (MW= 246.47 g/mol) | 100x | 9.9 g/L | 10 ml |
| 0.6 | mM$Ca(NO_3)_2$ (MW=236.2 g/mol) | 100x | 14.7 g/L | 10 ml |
| 5.0 | mM HEPES (MW= 238.3 g/mol) pH 7.2 | 100x | 119.1 g/L | 10 ml |

Use 30% Danieau's for incubation of dechorionated embryos

Use full strength Danieau's for surgery

Egg Water (EW)

A simple saline for embryos and larvae:

Dissolve 300 mg of Instant Ocean Salt mix per liter deionized water.

(Use reversed osmosis water, if available)

EW Methyleneblue

Use 0.5 to 2 ppm solution of methyleneblue in eggwater.

Inhibits fungal and bacterial growth

Embryo Medium EM (formerly used for embryo incubation. We find 30% Danieau's is better for survival of emryos)

1.0 ml Hank's Stock #1
0.1 ml Hank's Stock #2
1.0 ml Hank's Stock #4
95.9 ml dd $H_2O$
1.0 ml Hank's Stock #5

Use about 10 drops 1 M NaOH to pH 7.2

Fix Buffer

Dilute 2X fix buffer 1:1 with $dH_2O$

2X Fix Buffer

8.0 g Sucrose
0.15 ml 0.2M $CaCl_2$
90 ml 0.2M $PO_4$ buffer, pH 7.3

Check pH. Adjust if necessary to 7.3 with 1 M NaOH or HCl.
Add 0.2 M $PO_4$ buffer to a final volume of 100 ml

Fixatives

Fix buffer: 4% sucrose, 0.15 mM $CaCl_2$, 0.1 M $PO_4$ pH 7.3

For general fixation: 1.5% glutaraldehyde, 0.5% paraformaldehyde in fix buffer .
For antibody staining: 4% paraformaldehyde in fix buffer

Gelatin Embedding Medium

17% gelatin in 10% Hank's saline

Giemsa:

4 ml Giemsa Stock (Sigma Diagnostics)
4 ml 0.5 M Na Phosphate pH 7
200 mls distilled water

Hank's full strength with carbonate (Final): used for sperm storage only; not for embryos

9.9 ml Hank's Premix

0.1 ml Stock #6

Hank's (Full Strength) composition

0.137 M NaCl
5.4 mM KCl
0.25 mM $Na_2H\ PO_4$
0.44 mM $KH_2\ PO_4$
1.3 mM $CaCl_2$
1.0 mM $Mg\ SO_4$
4.2 mM $NaH\ CO_3$

Hank's Premix:

Combine the following in order:

10.0 ml Solution #1
1.0 ml Solution #2
1.0 ml Solution #4
86.0 ml $ddH_2O$
1.0 ml Solution #5

Store Hank's Premix in the refrigerator along with the Hank's solutions

Hank's Stock Solutions

Stock #1

8.0 g NaCl
0.4 g KCl
in 100 ml dd $H_2O$

Stock #2

0.358 g $Na_2HPO_4$ anhydrous
0.60 g $KH_2PO_4$
in 100 ml $ddH_2O$

Stock #4

0.72 g $CaCl_2$
in 50 ml $ddH_2O$

Stock #5

1.23 g $MgSO_4x7H_2O$
in 50 ml dd $H_2O$

Stock #6

0.35 g $NaHCO_3$

10.0 mls dd $H_2O$

MESAB: see tricaine, mesab

PO4 buffer (0.1 M, pH 7.3):

80 ml 0.1 M $Na_2HPO_4$ (13.8 g $Na_2HPO_4xH_2O$/liter $dH_2O$)

20 ml 0.1 M $NaH_2PO_4$ (26.8 g $NaH_2PO_4x7H_2O$/liter $dH_2O$)

Pronase E:

5 mg/ml pronase E diluted to 1 mg/ml in 30% Danieau's

PTU:

0.003% 1-phenyl-2-thiourea in 10% Hank's saline.

Ringer's Solutions:

Normal

116 mM NaCl

2.9 mM KCl

1.8 mM $CaCl_2$

5 mM HEPES, pH 7.2.

High calcium

116 mM NaCl

2.9 mM KCl

10 mM $CaCl_2$

5 mM HEPES, pH 7.2.

Calcium free

116 mM NaCl

2.9 mM KCl

5 mM HEPES, pH 7.2.

Subbing Solution for Slides:

1.5% gelatin

0.15% $CrK(SO_4)x2H_2O$.

TBS:

20 mM Tris-Cl, pH 7.5

500 mM NaCl

Tricaine / MESAB:

Tricaine (3-amino benzoic acidethylester) comes in a powdered form from Sigma (Cat.# A-5040). Make tricaine solution for anesthetizing fish by combining the following in a glass bottle with a screw cap:

400 mg tricaine powder
97.9 ml DD water
~2.1 ml 1 M Tris (pH 9).

Adjust pH to ~7. Store this solution in the freezer. (Buy the smallest amount possible because tricaine gets old.)

To use tricaine as an anesthetic combine the following in a 250 ml beaker:
4.2 ml tricaine solution
~100 ml clean tank water.

**Medaka**

Balanced Salt Solution (BSS) 10x (Yamamoto's isotonic BSS; Ringer´s solution):

| | |
|---|---|
| NaCl | 7.5 g |
| KCl | 0.2 g |
| $CaCl_2$ | 0.2 g |
| $NaHCO_3$ | 0.02 g |

to a final volume of 100 ml with distilled water; adjust pH to 7.3

Balanced Salt Solution (BSS) (for cell culture):

Solution A:

| For 500ml | |
|---|---|
| NaCl | 65g |
| KCl | 4g |
| $MgSO_4 \times 7H_2O$ | 2g |
| $CaCl_2 \times 2H_2O$ | 2g |
| Phenol red | 5mg |

Solution B:

5% $NaHCO_3$ in distilled water (Sterilize by filtration.)

Dilute 25ml of solution A with 475ml of distilled water and autoclave. Add 1 ml of solution B to this solution sterilized by filtration.

BSS-MB: add 1 ml 0.2% methylene blue per 1l BSS

Bleach (0.5%):

dilute 5% Na-hypochlorite (NaOCl; Sigma) in PBS (autoclaved)

Chelex solution (for fin-clips):

20% (w/v) Chelex 100 Sodiumform (Biorad # 143-2832) in dd$H_2O$; equilibrate for 12 hrs, then autoklave

CMF-PBS 90% (for cell culture):

| | |
|---|---|
| PBS (Dulbecco's Formula without Magnesium and Calcium),TaKaRa | 1 pellet |
| Distilled water | 111 ml |

To adjust to osmotic pressure of fish, $Ca^{++}$ and $Mg^{++}$ free PBS is diluted to 90% with distilled water.

DNA extraction buffer (AP-PCR):

| | Stock soln | Running soln (10ml) | Final conc |
|---|---|---|---|
| NaCl | 5 M | 0.2ml | 100mM |
| Tris(pH8.0) | 2 M | 0.1ml | 20mM |
| EDTA-2Na (pH8.0) | 0.5M | 1.0ml | 50mM |
| SDS | 10% | 0.5ml | 0.5% |
| Proteinase K | 20mg/ml | 0.2ml | 0.4mg/ml |

make up to 10ml with dd$H_2O$

DNA solution (cytoplasma injection):

| | | |
|---|---|---|
| 2 | µg | plasmid DNA |
| 4 | µl | 2.5 % phenol red |
| 4 | µl | 10x Ringer's |
| ad 40 | µl | $H_2O$ |

DNA solution (germinal vesicle injection):

| | | |
|---|---|---|
| 0.5 | µg | plasmid DNA |
| 5 | µl | 2.5 % phenol red |
| 5 | µl | 10x Ringer's |
| ad 50 | µl | $H_2O$ |

Electrophoresis solutions (AP-PCR):

10x TBE buffer (0.89M Tris-borate, 0.002M EDTA)

| | |
|---|---|
| Tris base | 324g |
| boreic acid | 165g |
| 0.5M EDTA(pH8.0) | 120ml |
| DW | to 3000ml |

7M urea-5% polyacrylamide gel (gel size:40cm x 20cm x0.35mm)

| | |
|---|---|
| urea | 13.5g |
| x10 TBE | 3ml |
| 40% acrylamide solution | 3.75ml |
| 10% APS | 180µl |
| DDW | to 30ml |
| TEMED | 26µl |

Formamide dye

| | |
|---|---|
| formamide | 10ml |
| 0.5M EDTA(pH8.0) | 0.2ml |
| BPB | 10mg |
| XC | 10mg |

Embryo Rearing Medium (ERM):

0.1% (w/v) NaCl, 0.003% (w/v) KCl, 0.004% (w/v) $CaCl_2$ x 2 $H_2O$, 0.016 % (w/v) $MgSO_4$ x 7$H_2O$, 0.0001% (w/v) methylene blue

ESM1 (for blastula cell culture):

13 g/l DMEM (4.5 g glucose/l; Gibco)
20 mM Hepes
50 µg/ml streptomycin-50 U/ml penicillin
1 X Non-essential amino acids (1 mM; Gibco)
1 X Na-pyruvate (1 mM; Gibco)
1 X Na-selenite (2 nM; Gibco)
1 X glutamine (2 mM)
2 X 2-mercaptoethanol (100 µM; Gibco)
15% ES-FCS (Gibco)
1% rainbow trout serum (self made)
10 ng/ml bFGF; human recombinant (Gibco)
10 ng/ml LIF; human recombinant (UPI)
Medaka embryo extract (1 embyo per ml medium; self made)

Gelatin (0.1%):

0.5 g gelatin (Type A, 300 Bloom; Sigma) in 500 ml water, autoclave

Lysis buffer (for fin-clips):

100mM NaCl; 1.2% sarcosyl in $ddH_2O$, autoclaved

**medaka PBS 10x::**

**1M NaCl, 19.5mM KCl, 59mM $Na_2HPO_4$, 11mM $KH_2PO_4$; pH to 7.3 - 7.4**

medaka PBS (for cell culture):

108 mM NaCl; 252 mM $Na_2HPO_4$; 22.5 mM $KH_2PO_4$; pH7.3, autoclave

Oocyte Medium (Earle's medium 199):

| | |
|---|---|
| 9.9g | Medium 199, Earle's salts |
| 2.2g | $NaHCO_3$ |
| 50mg | penicillin |
| 30mg | streptomycin |
| | (or: 5ml Pen/Strep solution; Gibco) |
| 20g | BSA |

ad 1.25l $ddH_2O$; add NaOH to adjust pH to 8.0; filter through 0.2µm filter

PCR buffers (AP-PCR):

| | x10 PCR buffer (low stringency) | x10 PCR buffer (high stringency) |
|---|---|---|
| KCl | 50mM | 50mM |
| Tris(pH7.6) | 10mM | 10mM |
| gelatin | 0.01% | 0.01% |
| MgCl2 | 5mM | 1.5mM |

TM buffer (for cell culture):

100 mM NaCl
5 mM KCl
5 mM Hepes
1 mM $CaCl_2$
1 mM $MgCl_2$
100 µg/U/ml Strep-Peni
pH7.0; filter through 0.2 µm filter

TM1 buffer:

100 mM NaCl
5 mM KCl
5 mM Hepes
pH7.0; filter through 0.2 µm filter

TM-5% PEG:

Proteinase K (10 mg/ml in water)
1 vol 2 X TM
1 vol 10% PEG
filter through 0.2 µm filter

## Teachers

**Chris Amemiya**
Virginia Mason Rersearch Center
Benaroya Research Institute
Molecular Genetics Dept.
1201 Ninth Avenue
Seattle, WA 98101 USA
phone.: +1 206-583-6525 (office);
-6400 (lab);
fax: +1 206-583-2297
e-mail: camemiya@vmresearch.org

**Wolfgang Driever**
Institut für Biologie I
Entwicklungsbiologie
Hauptstr. 1
79104 Freiburg
GERMANY
fax: +49-761-203-2598
E-mail: driever@sun2.ruf.uni-Freiburg.de

**Thorsten Henrich**
ERATO,
Kondoh differentiation signaling project,
Kinki-chihou Hatsumei Center Building,
Yoshida-Kawaramachi 14, Sakyo-ku,
Kyoto 606-8305, Japan
email: henrich@dsp.jst.go.jp
phone +81-75-771-9362
fax +81-75-771-8281
e-mail: henrich@dsp.jst.go.jp

**Laure Bally-Cuif**
Zebrafish Neurogenetics Group
GSF-Research Center
Institute of Developmental Genetics
Ingolstaedter Landstrasse 1
D.85764 Neuherberg Germany
phone. +49 89 3187.3562 (office)
+49 89 3187.2944 (lab)
fax. +49 89 3187.3099
e-mail: bally@gsf.de

**Makoto Furutani-Seiki**
Kondoh Differentiation Signaling Project
JSTC
14 Kawara-cho Yoshida, Sakyo-ku
Kyoto
606-8394 Kyoto, JAPAN
phone: +81-75-771-9362
fax: +81-75-771-8283
e-mail: furutaniseiki@msi.biglobe.ne.jp

**Yunhan Hong**
Dept of Biological Sciences
National University of Singapore
14 Science Drive 4
Singapore 117543, Singapore
fax: 7792 486
e-mail: dbshyh@nus.edu.sg

**Don Kane**
Department of Biology
University of Rochester
Box 270211
Rochester, NY 14627
USA
fax: +1-716-275-2070
E-mail: ddke@uhur

**Felix Loosli**
Developmental Biology Programme
EMBL Heidelberg
Meyerhofstrasse 1
D-69117 Heidelberg
GERMANY
phone: +49 6221-387- 147
fax: +49 6221-387- 166
e-mail:
Felix.Loosli@EMBL-Heidelberg.de

**Hiroshi Mitani**
Department of Integrated Biosciences
Department of Biological Sciences
University of Tokyo
5-1-5 Kashiwanoha
Kashiwa City 277-8562, Japan
phone: +81 3 5841-4441
fax: +81 3 5802 2911
e-mail: Mitani@k.u-tokyo.ac.jp

**Indrajit Nanda**
Experimentelle und Klinische Zytogenetic
Biozentrum der Universität Würzburg
Institut für Humangenetik
Am Hubland
97074 Würzburg
phone: +49 931 888 - 4078
fax: +49 931 888 – 4069
e-mail: ijn@biozentrum.uni-wuerzburg.de

**Kyoshi Naruse**
Department of Biological Sciences
Graduate School of Science
University of Tokyo
Hongo7-3-1, Bunkyo-ku
Tokyo 113-0033, Japan
phone:+81-3-5841-4443
FAX: +81-3-5841-4410 (office)
e-mail: naruse@biol.s.u-tokyo.ac.jp

**Carl Neumann**
Developmental Biology Programme
EMBL Heidelberg
Meyerhofstrasse 1
D-69117 Heidelberg
GERMANY
phone: +49 6221-387- 603
fax: +49 6221-387- 166
e-mail:
Carl.Neumann@EMBL-Heidelberg.de

**Masaru Nonaka**
Department of Biological Sciences
Graduate School of Science
University of Tokyo
7-3-1 Hongo, Tokyo 113-0033 Japan
phone: +81-3-5841-7589
fax:+81-3-5800-3397
e-mail: mnonaka@biol.s.u-tokyo.ac.jp

**Manfred Schartl**
Physiological Chemistry I
Biocenter of the University Würzburg
Am Hubland
97074 Würzburg
GERMANY
phone: +49-931-888-4148
fax: +49-931-888-4150
e-mail:
phch1@biozentrum.uni-wuerzburg.de

**Akihiro Shima**
Department of Integrated Biosciences
Department of Biological Sciences
The University of Tokyo
5-1-5 Kashiwanoha
Kashiwa City 277-8562, Japan
phone: +81 3 5841-4441
fax: +81-3-5802 2911
e-mail: shima@k.u-tokyo.ac.jp

**Lilianna Solnica-Krezel**
Department of Biological Sciences
Vanderbilt University
VU Station B 351634
Nashville, 37235-1634
USA
phone. +1 615-343-9413
fax. +1 615-343-6707
e-mail:
lilianna.solnica-krezel@vanderbilt.edu

**Monte Westerfield**
Institute of Neuroscience
University of Oregon
219 Huestis Hall
Eugene OR 97403, USA
phone: +1 541 346 4607
fax: +1-541-346-4548
e-mail: Monte@UONEURO.uoregon.edu

**Christoph Winkler**
Physiological Chemistry I
Biocenter of the University Würzburg
Am Hubland
97074 Würzburg
GERMANY
phone: +49-931-888-4142
fax: +49-931-888-4150
e-mail:
cwinkler@biozentrum.uni-wuerzburg.de

**Jochen Wittbrodt**
Developmental Biology Programme
EMBL Heidelberg
Meyerhofstrasse 1
D-69117 Heidelberg
GERMANY
phone: +49 6221-387- 576
fax: +49 6221-387- 166
e-mail:
**Jochen.Wittbrodt@EMBL-Heidelberg.de**

Wittbrodt Lab: Alexander Arenz, Matthias Carl, Filippo DelBene, Clemens Grabher, Erika Grzebisz, Annette Krone, Felix Loosli, Lucia Poggi, Rebecca Quiring, Martina Rembold, Beate Wittbrodt. E-mail: <first name>.<last name>@EMBL-Heidelberg.de

Additional help by

Antonio Giraldez (Giraldez@EMBL-Heidelberg.de), Juan-Ramon Martinez-Morales (isrr348@cajal.csic.es), Marlene Rau (M.Rau@DKFZ.de), Matthias Schaefer (M.Schaefer@Biozentrum.uni-wuerzburg.de).

## Participants


**Massimiliano Andreazzoli**
Lab. Biol. Cell. e Sviluppo
Fisiologia e Biochimica
Via Carducci, 13
56010 Ghezzano (Pisa)
Italy
phone.: +39 050 878356
fax: +39 050 878486
Email: andream@dfb.unipi.it

**Anja Berger**
MPI for Molecular Genetics
Lehrach
Ihnestr. 63-73
14195 Berlin
Germany
phone.: +49 30 84131339
fax: +49 84131128
Email: berger_a@molgen.mpg.de

**Karin Dumstrei**
University of California Los Angeles
Molecular Cell Developmental Biology
4214 Life Science Bldg
621 Charles E. Young Dr. South
CA 90095 Los Angeles
United States
phone.: (310) 206 7376
fax: (310) 206 3987
Email: karind@ucla.edu

**William Jones**
University of Konstanz
Evolutionary Biology
Universitätsstra e 10
D-78457 Konstanz
Germany
phone.: +49 7531 883710
fax: +49 7531 883018
Email: william.jones@uni-konstanz.de

**Ronja Bahadori**
Brain Research Institute, ETH Zurich
Biology
Winterthurerstr. 190
8057 Zurich
Switzerland
phone.: +41 1635 3228
fax: +41 1635 3303
Email: bahadori@hifo.unizh.ch

**Miguel Concha**
University College London
Anatomy and Developmental Biology
Gower Street
WC1E 6BT London
United Kingdom
phone.: 44 207 6793331
fax: 44 207 6797349
Email: m.concha@ucl.ac.uk

**William Jackman**
University of Colorado at Boulder
Evolutionary, Population, and Organismic Biology
University of Colorado
Campus Box 334
80309-0334 Boulder
United States
phone.: +1 303 735 5153
fax: +1 303 492 8699
Email: william.jackman@colorado.edu

**Astrid Keijser**
NIOB/ Hubrecht Laboratorium
group van Eeden
Uppsalalaan 8
3584 CT Utrecht
The Netherlands
phone.: +31 30 2121800
fax: +31 30 2516464
Email: astrid@niob.knaw.nl

**Michael Lang**
GSF Forschungszentrum fuer Umwelt und Gesundheit
Institut fuer Saeugetiergenetik - Zebrafischgenetik
Ingolstaedter Landstr. 1
85764 Neuherberg
Germany
phone.: +49 (0)89 3187-3181
fax: +49 (0)89 3187-3192
Email: michael.lang@gsf.de

**Jae-Seong Lee**
Hanyang University
Department of Environmental Science
17 Haengdang-dong, Seongdong-gu
133-791 Seoul
Korea, South
phone.: +82 2 2290 0769
fax: +82 2 2294 6270
Email: jslee2@hanyang.ac.kr

**Ming MA**
Institute of Molecular Agrobiology, National University of Singapore
1 Research Link, NUS, Singapore
117604 Singapore
Singapore
phone.: +65 6872 7094
fax: +65 6872 7007
Email: maming@ima.org.sg

**Csaba Miskey**
Max Delbruck Center for Molecular Medicine
Transposition Group
Robert Rossle Str. 10
13092 Berlin
Germany
phone.: +49 30 94062545
fax: +49 30 94062547
Email: cs.miskey@mdc-berlin.de

**Andrea Pasini**
The National Institute for Medical Research
Developmental Neurobiology
The Ridgeway, Mill Hill
NW7 7AA London
United Kingdom
phone.: +44 207 959 3666 ext 2253
fax: +44 208 906 4477
Email: apasini@nimr.mrc.ac.uk

**Jorge Pereira Pinto**
Universidade do Algarve
FCMA/CCMAR
Campus de Gambelas
8000 Faro
Portugal
phone.: +351 965357850
fax: +351 289 818353
Email: jpinto_1@aeiou.pt

**Ombretta Pozzoli**
Università degli Studi di Milano
Biologia
Via Celoria 26
20133 Milan
Italy
phone.: +39 02 503 14791
fax: +39 02 503 14781
Email: ombretta.pozzoli@unimi.it

**Michael Schebesta**
IMP
Dr. Bohrgasse 7
A-1030 Vienna
Austria
phone.: +43 1 79730 425
fax: +43 1 798 71 53
Email: schebesta@nt.imp.univie.ac.at