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Zebrafish (*Danio rerio*) as model system

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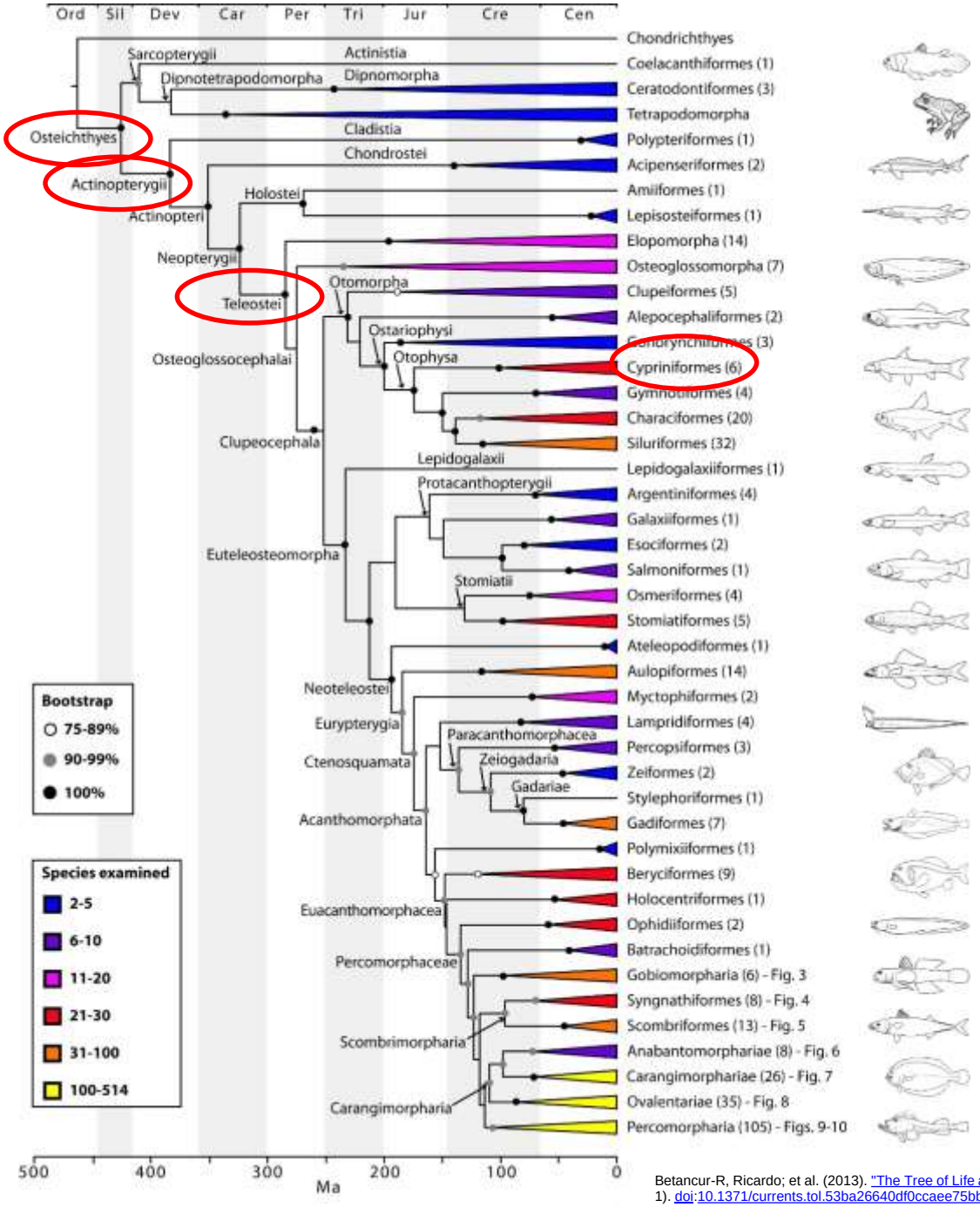


Taxon *Euteleostomi* (bony vertebrates, like human)

Superclass *Osteichthyes* (bony fish). This includes the tetrapods!

Class *Actinopterygii* (ray-finned fish, „Strahlenflosser“), tetrapods belong to the clade of lobe-finned fish (Sarcopterygii „Fleischflosser“)

Subclass *Teleostei* („echte Knochenfische“)

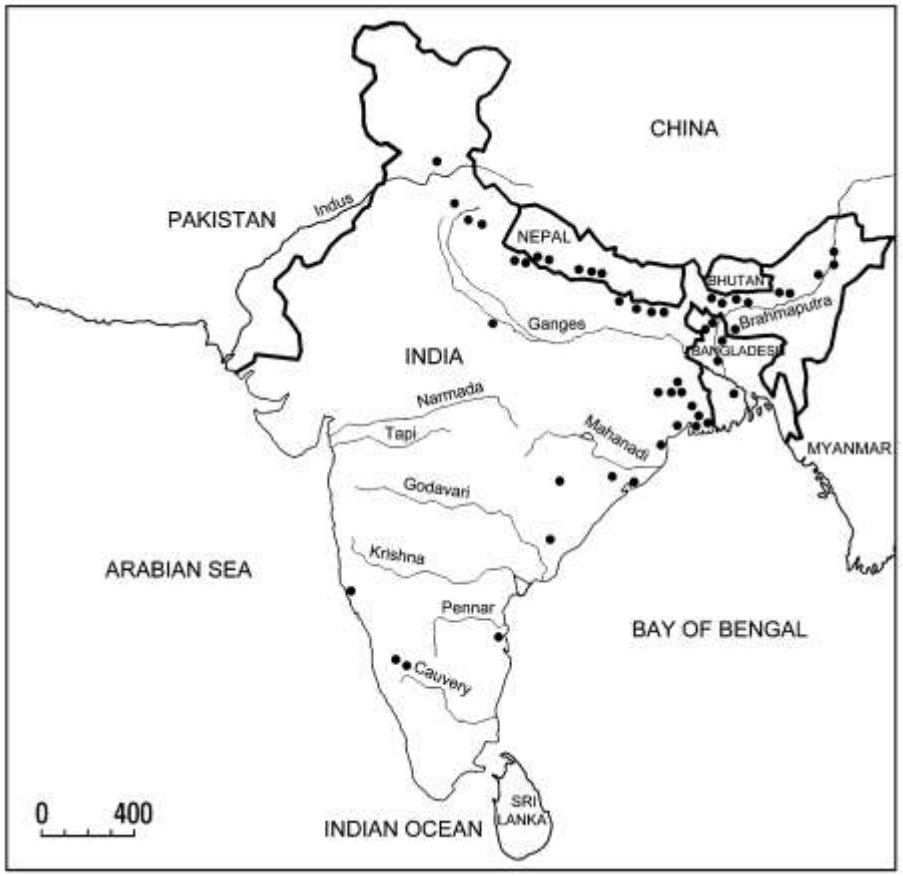
Order *Cypriniformes* („Karpfenfische“)

there are 44 species in the genus *Danio*

Betancur-R, Ricardo; et al. (2013). "The Tree of Life and a New Classification of Bony Fishes". *PLOS Currents Tree of Life* (Edition 1). doi:10.1371/currents.tol.53ba26640df0ccaee75bb165c8c26288.

Habitat

- freshwater fish
- indigenous to Indian subcontinent (India, Bangladesh, Nepal, Myanmar, Pakistan)
- „Danio" is derived from Bengali word for „in the rice field"
- prefer shallow, slow-flowing or still water (floodplains, ponds, rice fields)
- in the wild found at 16.5 to 33 °C, slightly alkaline pH (7.9-8.2)
- occopy the whole of the water column
- equally likely in open water and in vegetation
- form shoals
- life-span in the wild unclear



Spence et al. Biol. Rev. (2008), 83, pp. 13–34

Features

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- up to 40 mm in length (snout to base of caudal fin), up to 700 mg
- up to 3 years of age in the lab
- mouth pointing upwards, lower jaw protruding further than upper
- 5 to 7 dark stripes

- 2 pairs of fins: pectoral and pelvic
- 3 unpaired fins: dorsal, anal, caudal

Sexual dimorphism

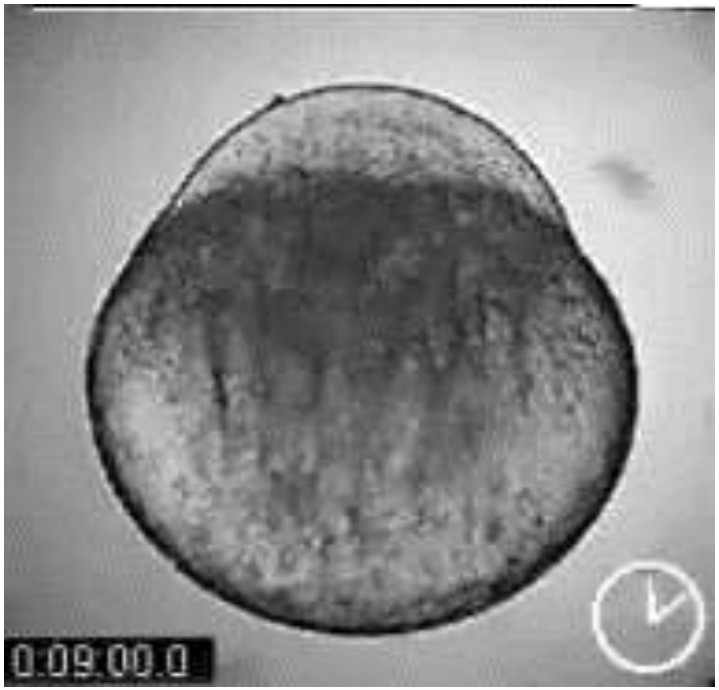
females:

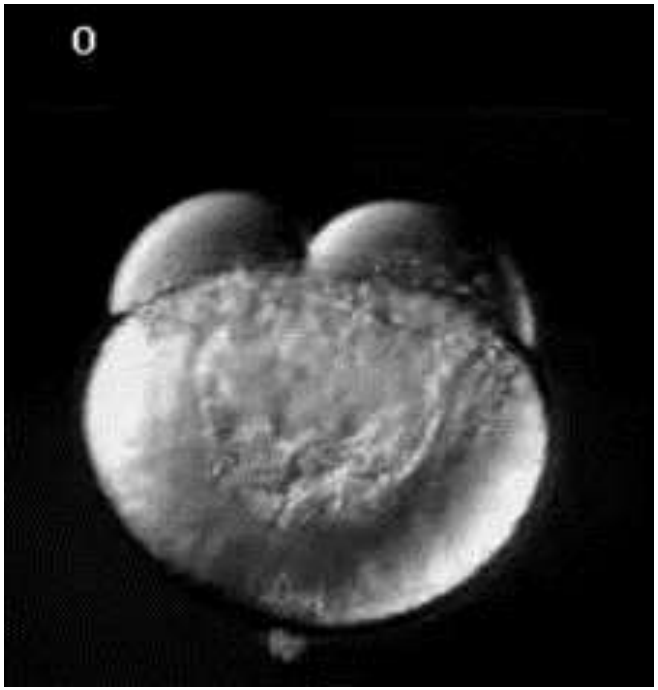
prominent belly (eggs)
interstripe color whitish
yellow dorsal fin

males:

slender body shape
interstripe color pinkish (most obvious at anal fin)
white dorsal fin

Imaging of early development





Imaging of early development

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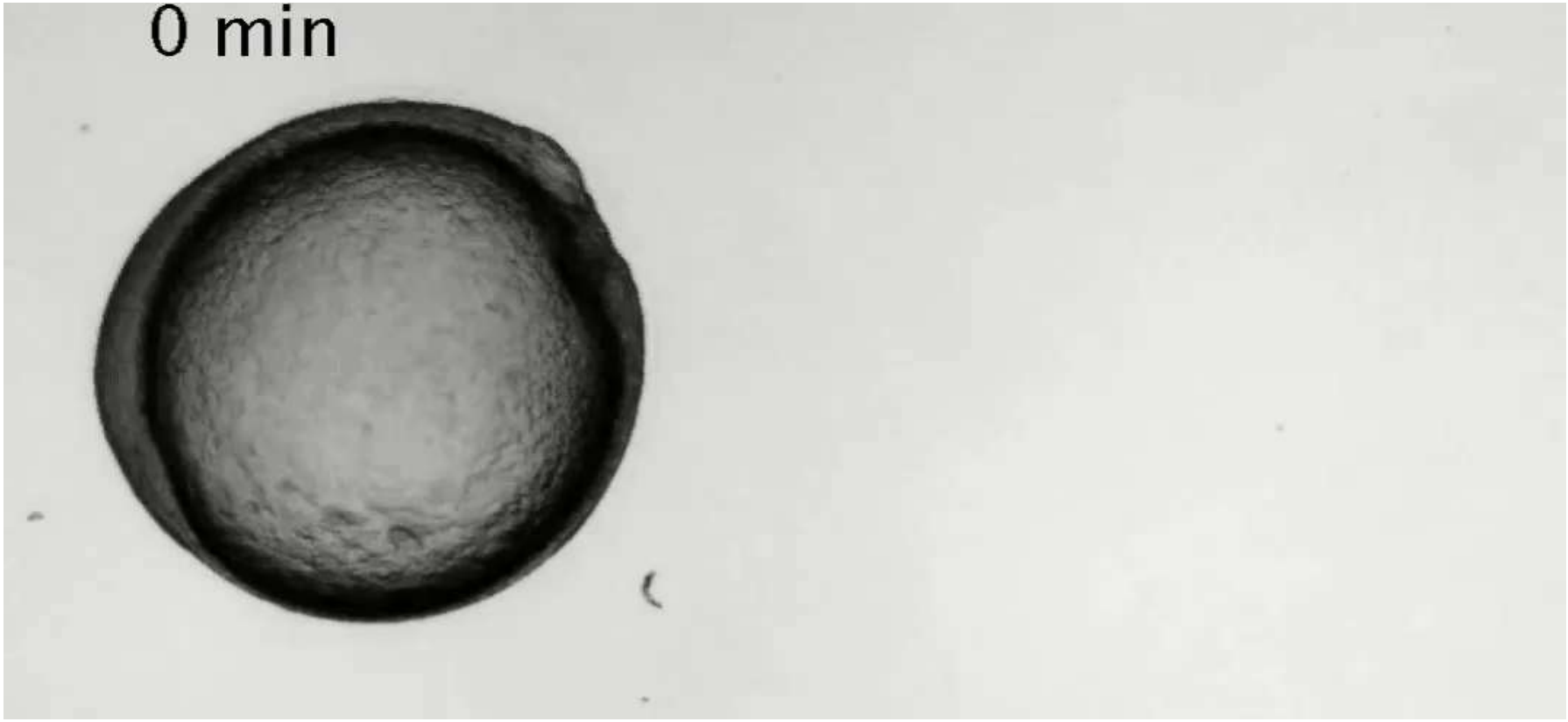
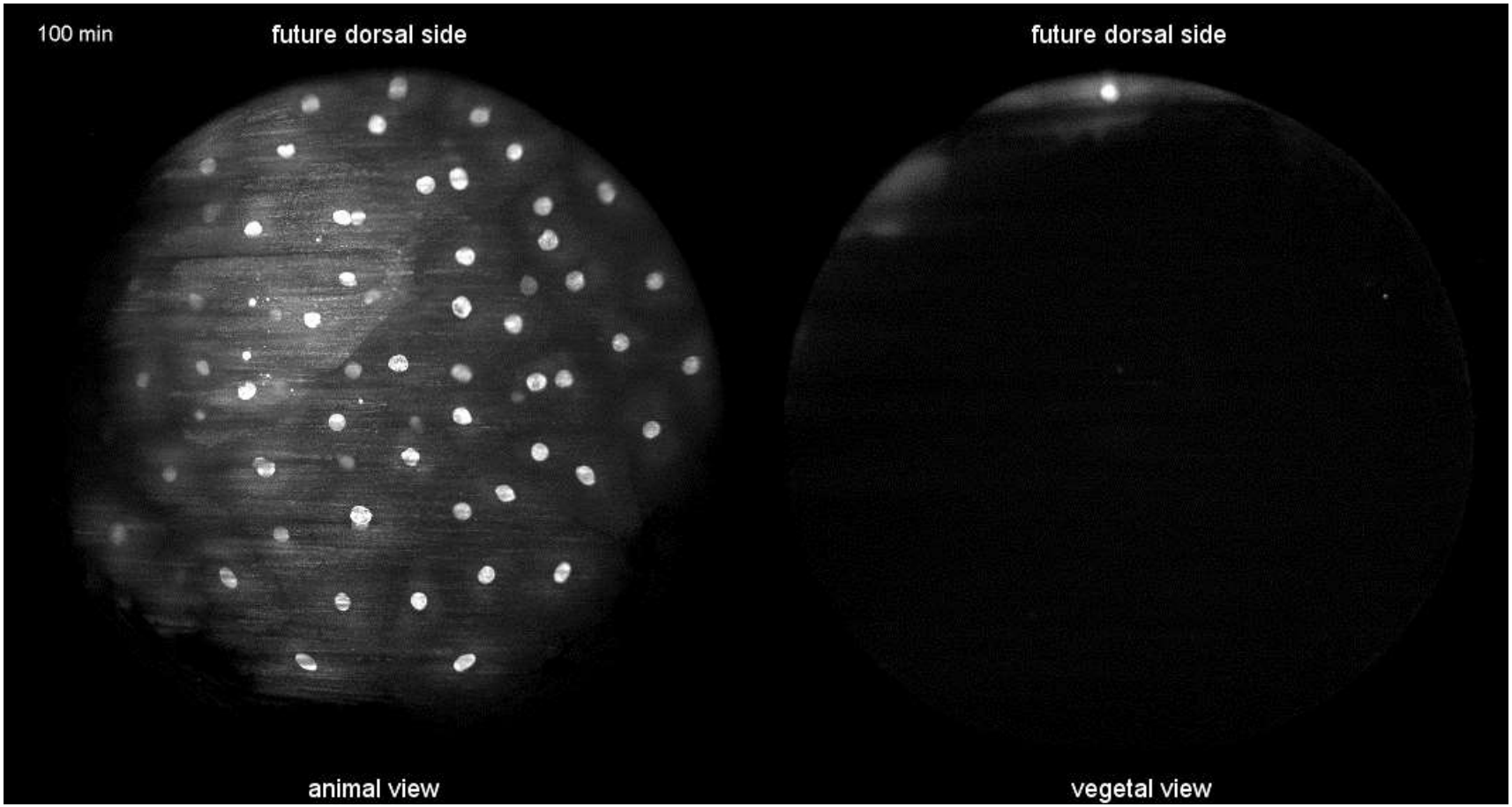


In toto imaging



Transparency of early embryos

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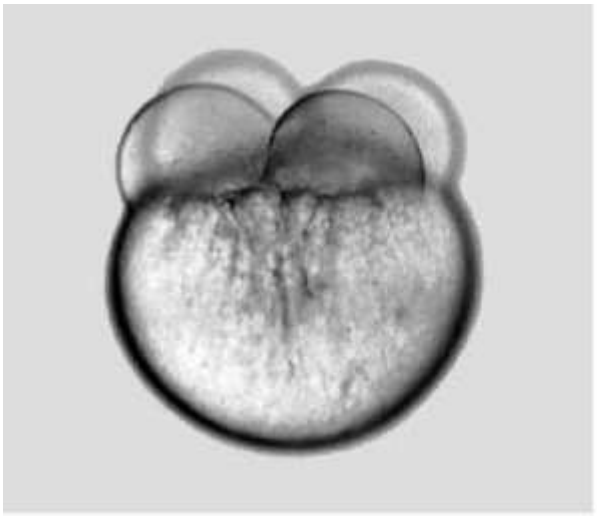
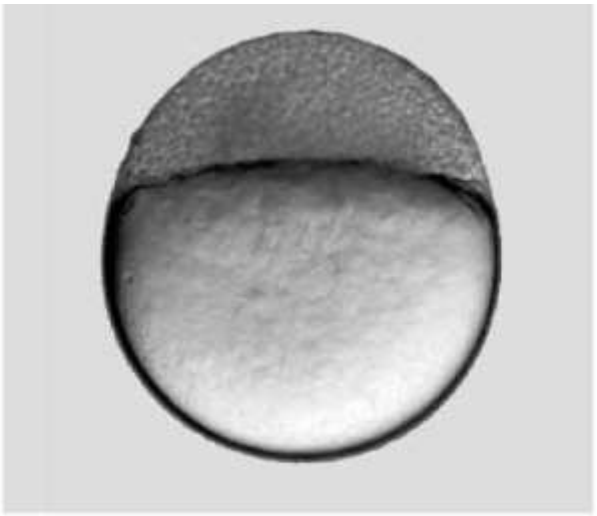
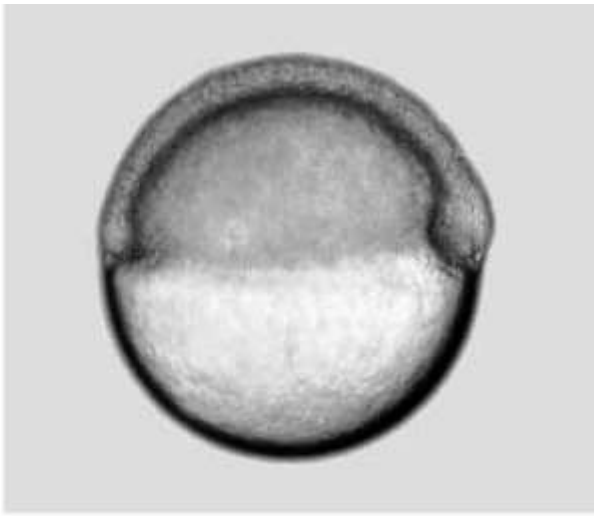


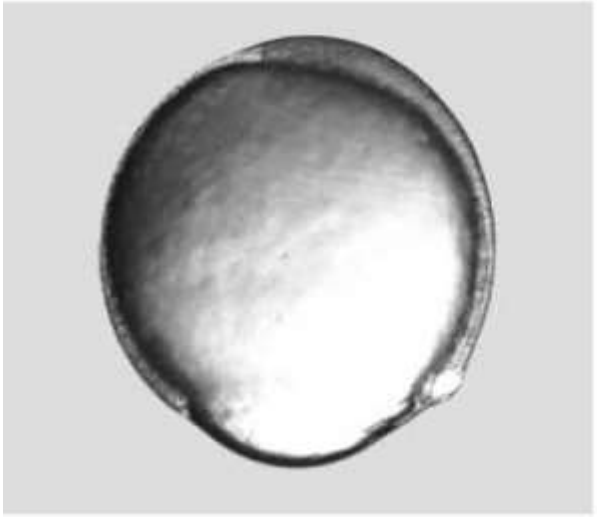
4-cells 1 h



sphere 4 h

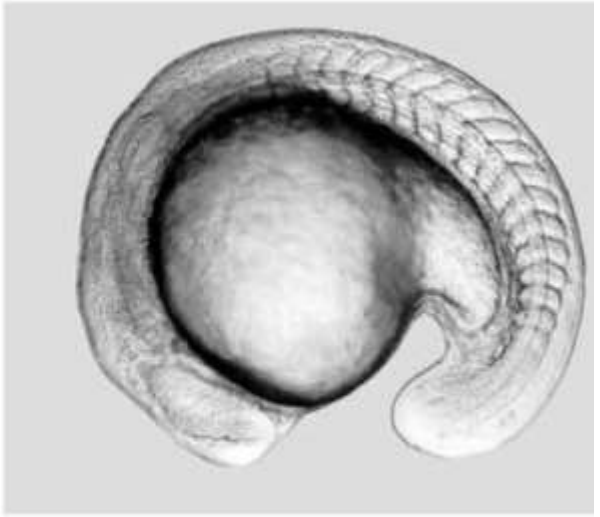


shield 6 h



80% epiboly 8 1/3 h

1 somite 10 1/3 h



19 somites 18 1/2 h

Transparency

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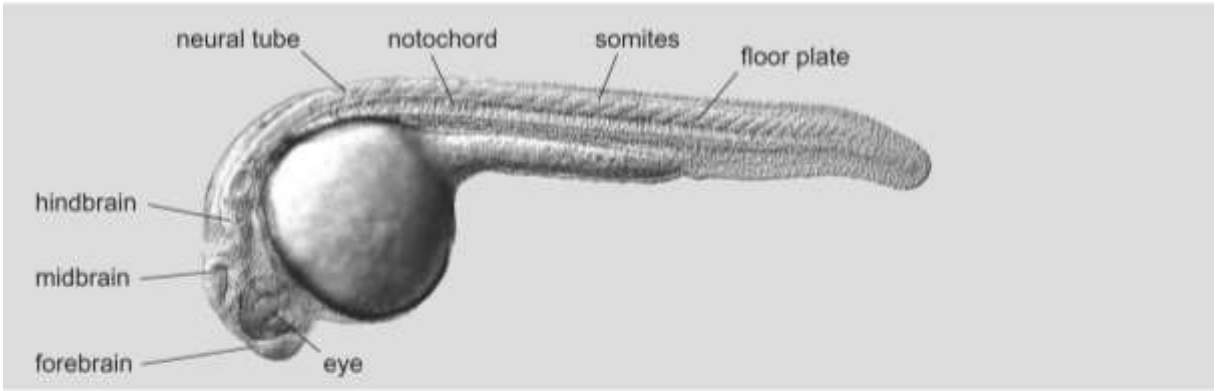
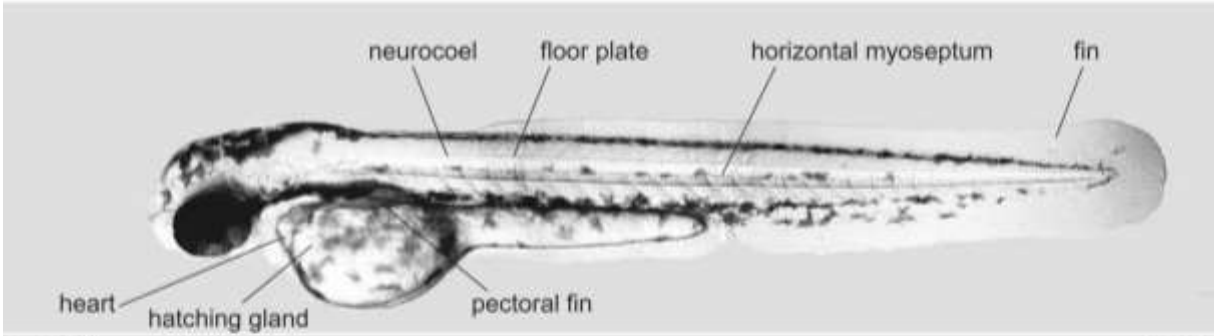
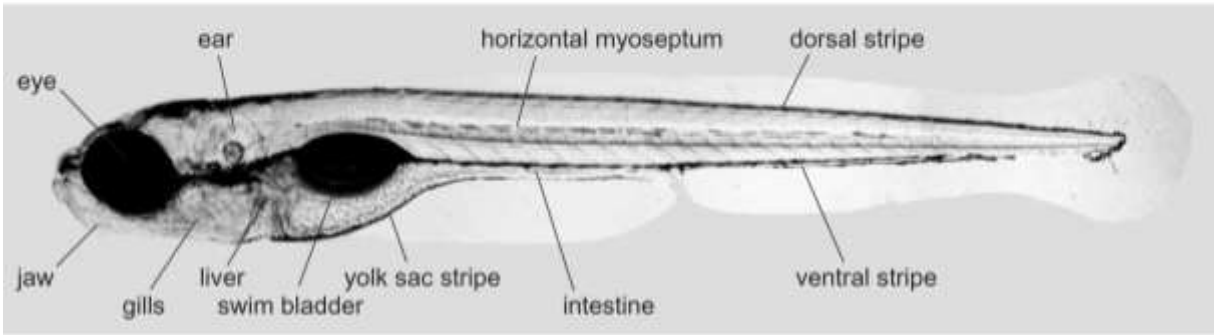


pharyngula period 29 h



hatching period 48 h



swimming larva 5 d

Design of ENU forward genetic screens

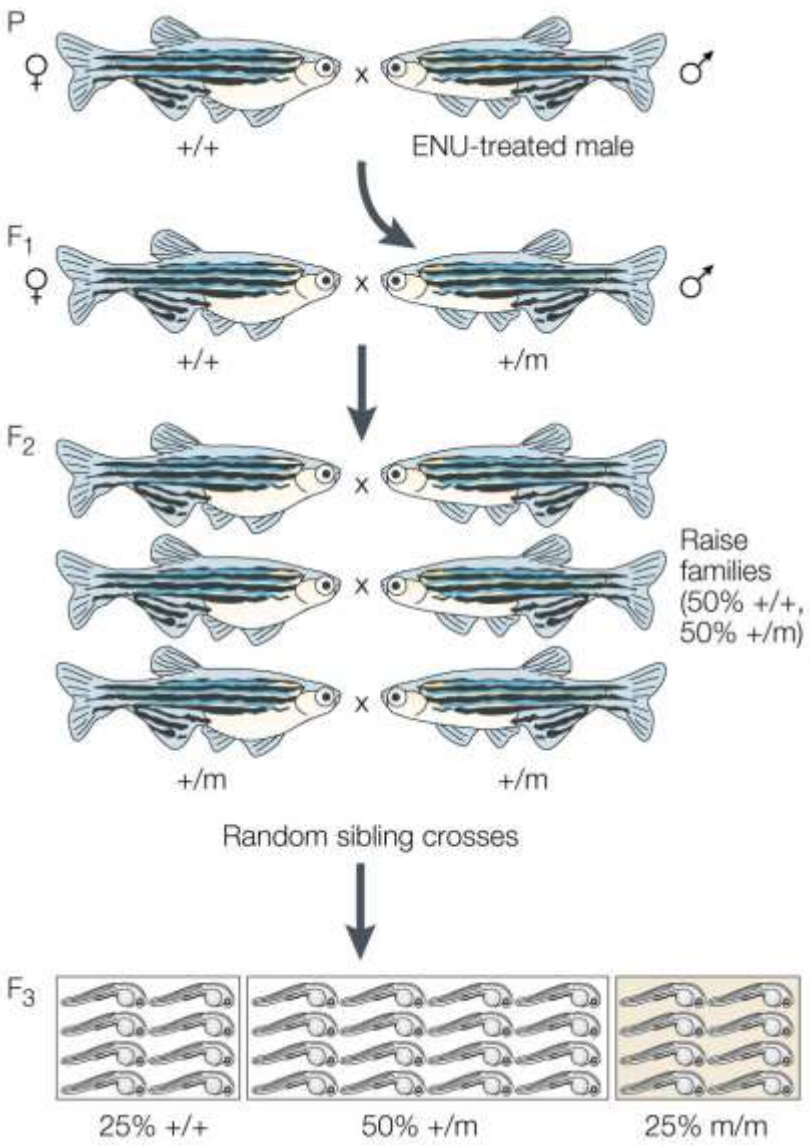


Figure 1 | **Outline of large-scale F_2 genetic screens.** In F_2 screens, a mutagen, such as ethylnitrosourea (ENU), is used to generate hundreds of point mutations in the male pre-meiotic germ cells (spermatogonia). ENU-treated males are crossed to wild-type females to produce the F_1 heterozygous progeny. F_1 fish are then crossed to siblings to create F_2 families, half of which are genotypically heterozygous for a specific mutation (m), whereas the other half are wild type. F_2 siblings are crossed, and the resulting F_3 progeny are 25% wild type (+/+), 50% heterozygous (+/m) and 25% homozygous (m/m) for a recessive mutation. Together, the Boston and Tübingen screens, starting from about 300 ENU founder males, involved raising more than 5,000 F_2 families, analysing more than 6,000 mutagenized genomes and selecting more than 2,000 new developmental mutants for characterization.

Examples of mutants found in forward screens

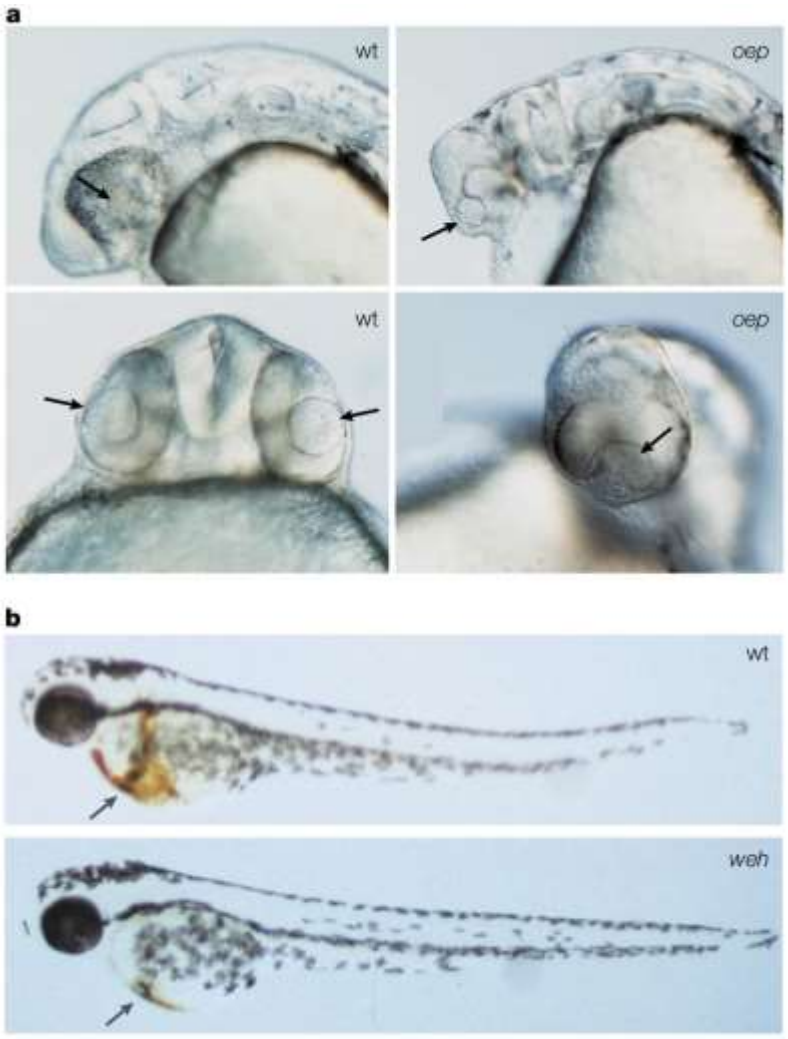


Figure 2 | **Examples of mutants identified in zebrafish large-scale screening efforts. a** | Mutants for *one-eyed pinhead* (*oep*), which encodes a member of the Nodal signalling pathway, lack endoderm, prechordal plate and ventral neuroectoderm, which results in severe cyclopia (arrows denote lens position) among other defects. Lateral (top panels) and anterior–ventral (bottom panels) view of wild type (wt) and *oep* mutants. Reproduced with permission from REF. 78 © (1996) Company of Biologists Ltd. **b** | The recessive embryonic-lethal mutation *weissherbst* (*weh*) results in hypochromic blood with decreasing blood cell counts. Staining of 2-day-old embryos with O-dianisidine to visualize haemoglobin (arrows) shows reduced levels of haemoglobin in the *weh* mutant. Reproduced with permission from REF. 15 © (1996) Company of Biologists Ltd. **c** | A dominant mutation, *hagoramo* (*hag*), which results in a disrupted stripe pattern of adult fish and encodes a protein with a possible role in proteolysis, was generated by insertional mutagenesis. Reproduced with permission from REF. 37 © (2000) Elsevier Science.

Examples for screens

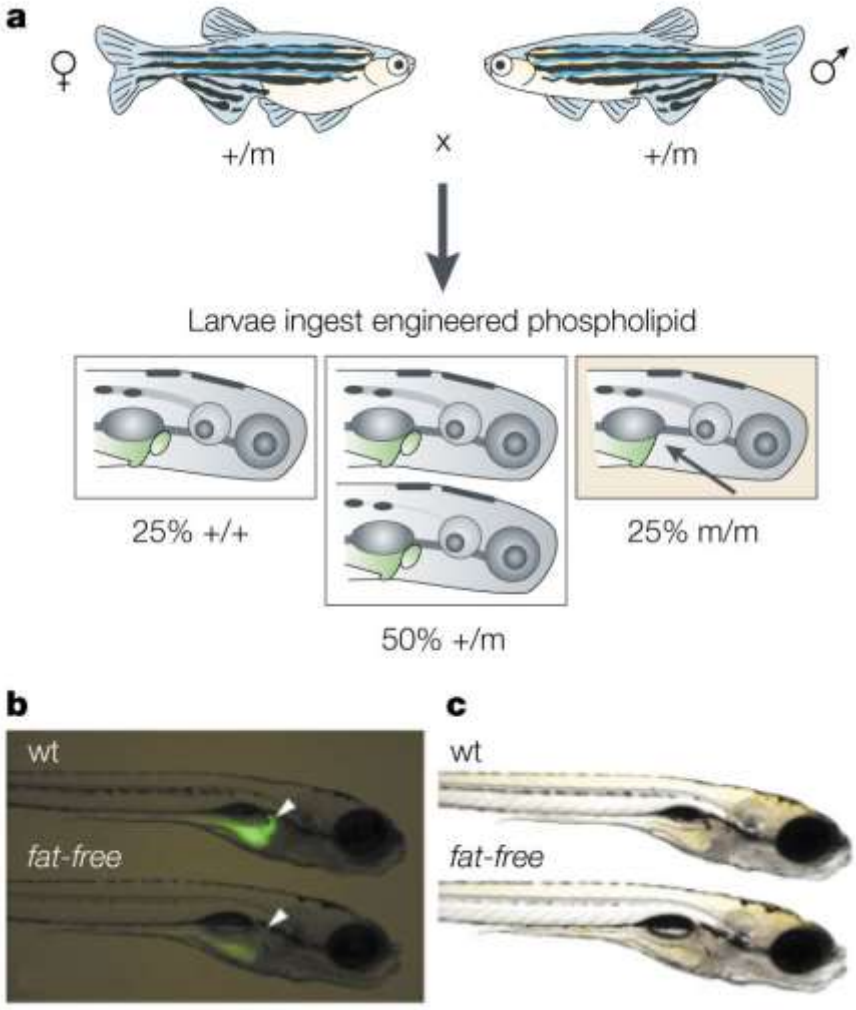


Figure 4 | **Fluorescent reporter screens. a** | Fluorescent reporters can be used to screen for mutations in specific enzymatic processes in live embryos[39]. For example, larvae derived from crossing two fish that are heterozygous for a specific digestive tract mutation (for example, *fat free*, see part **b**) ingest phospholipids that are engineered to fluoresce during normal lipid processing. One-quarter of the larvae are homozygous for a recessive, digestive-tract mutation and lack gall-bladder fluorescence (arrow), which indicates a defect in normal lipid processing. **b,c** | The larval digestive mutant *fat free* is defective for lipid processing. Wild type (wt) and *fat free* mutants were bathed in a type of phospholipid (PED6) that was engineered to fluoresce when cleaved by the phospholipase (PLA_2) enzyme (for details, see REF. 39). Wild-type day 5 larvae show intense gall-bladder fluorescence (arrowhead in **b**), whereas *fat free* day 5 larvae show severely reduced gall-bladder fluorescence. Note that under normal lighting conditions (**c**), the digestive tract appears normal in *fat free* mutant larvae. m, mutation. Panel **b** is reproduced with permission from REF. 39 © (2000) American Association for the Advancement of Science.

Examples for screens

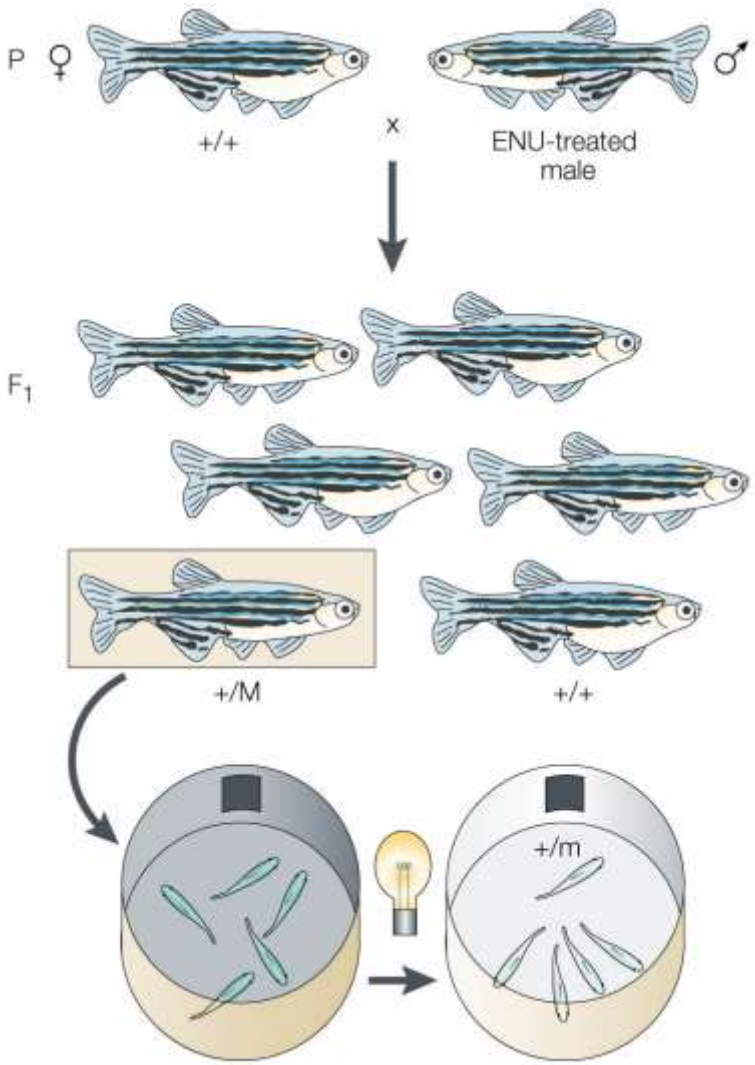


Figure 6 | **Behaviour screens: visual adaptation mutants.** A behavioural test can be a measure of visual sensitivity and be used to screen for subtle, eye-specific mutations in adult zebrafish. Adult F_1-generation fish derived from ethylnitrosourea (ENU)-mutagenized founders are placed in a transparent container that is surrounded by a rotating drum marked with a black square that represents a threatening object[49,50]. After initial dark adaptation, normal zebrafish rapidly 'escape' the threatening object in light above (but not below) their visual threshold (right drum). By contrast, fish that are heterozygous for the mutation *night blindness b* show the escape response at a visual threshold that is 2–3 log units above the average[50]. M, dominant mutation on an ENU-mutagenized chromosome.

Transgenesis

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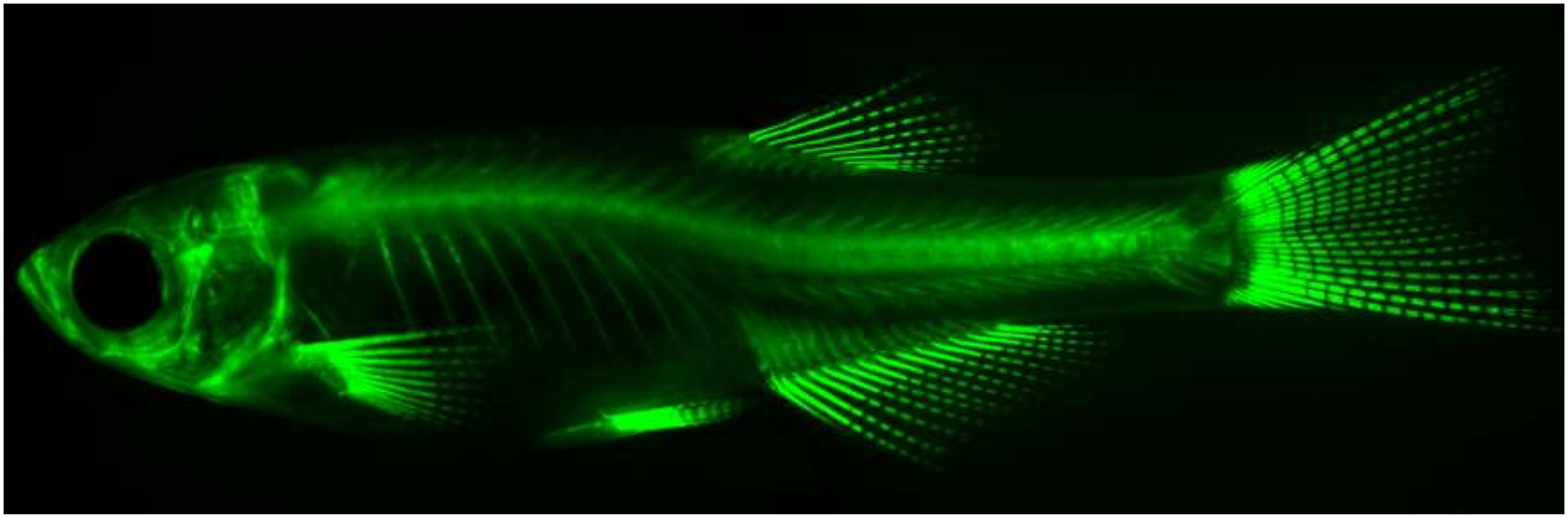

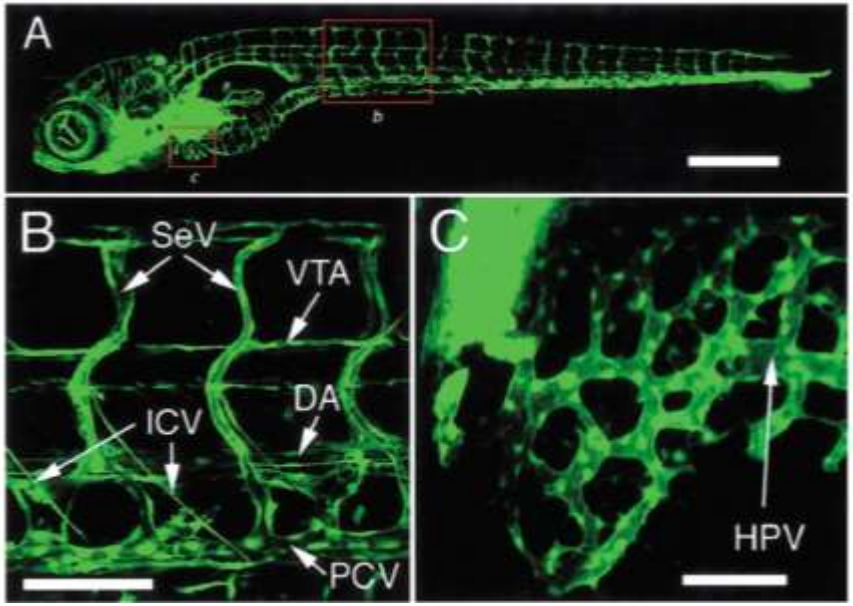

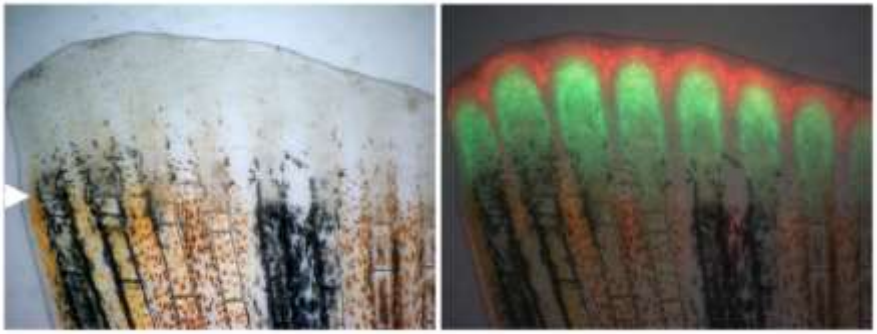

Transgenesis

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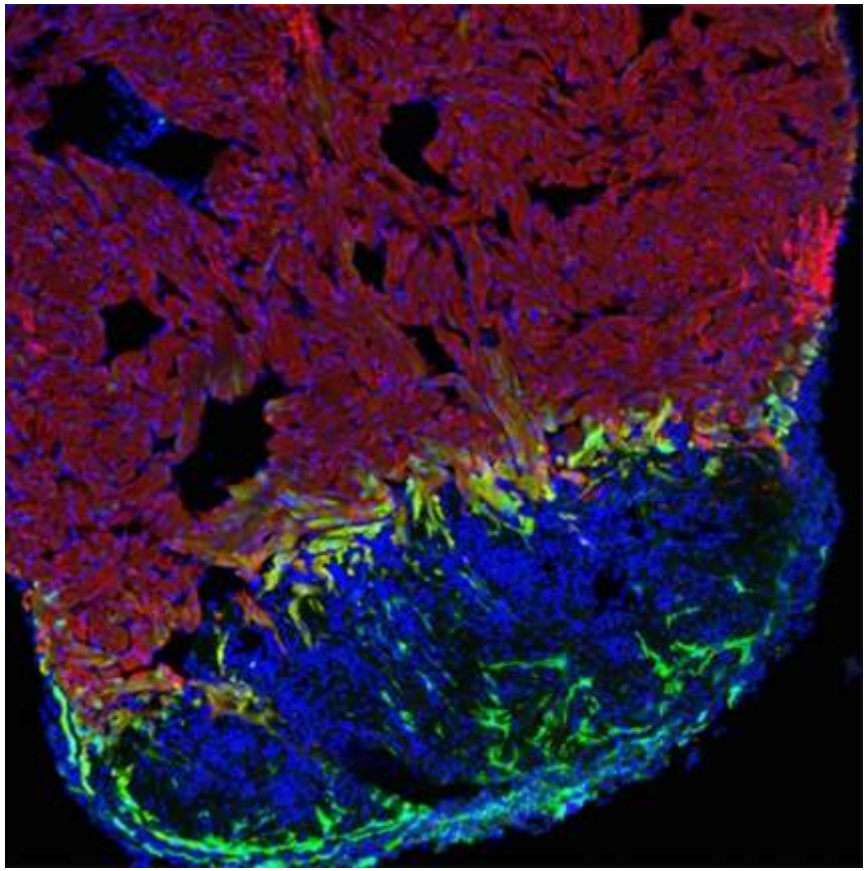
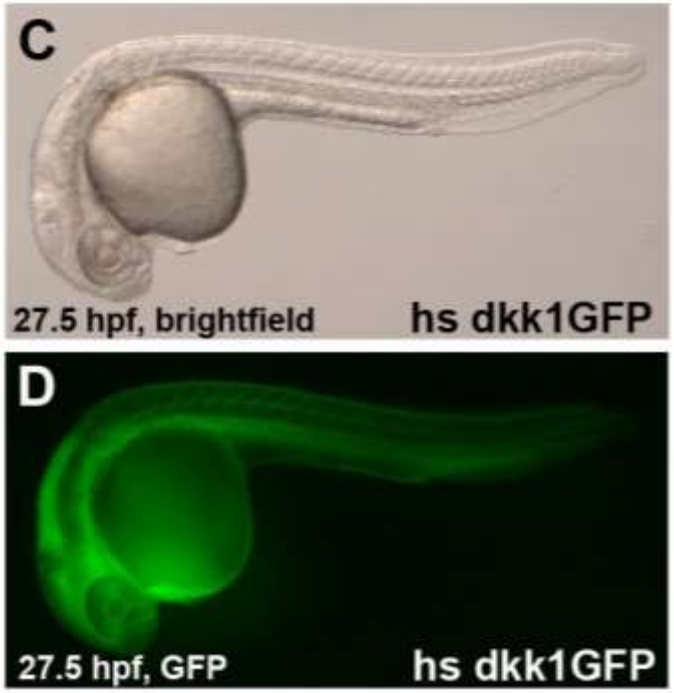
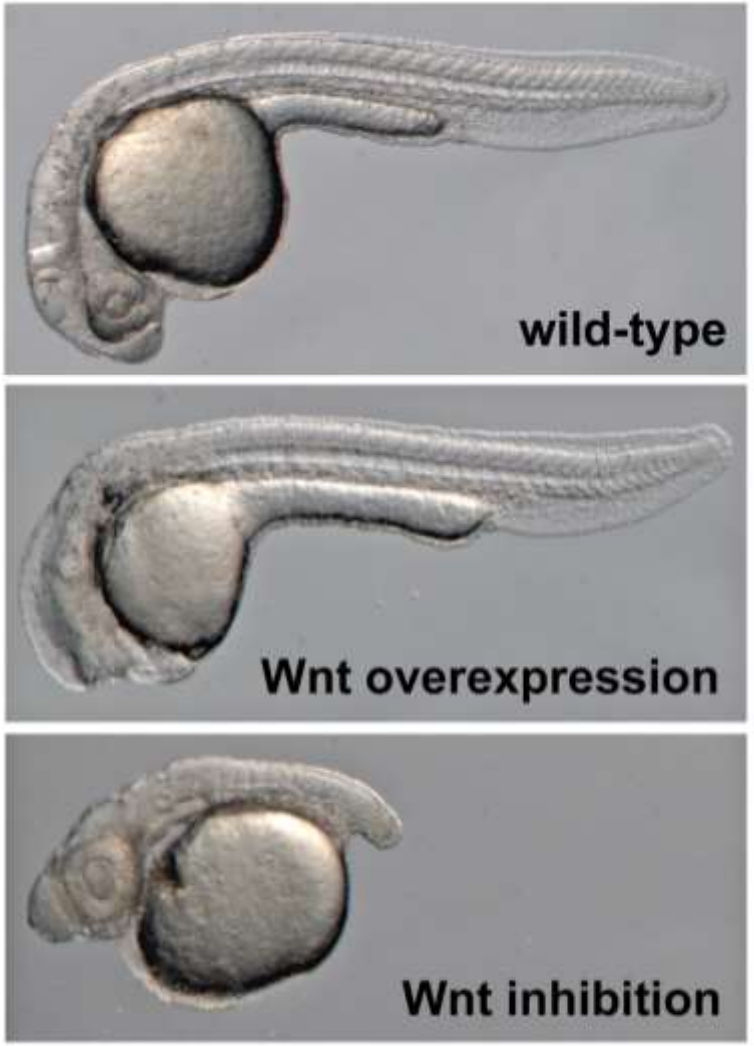


Transgenesis

hs @ 24 hpf for 2.5 h





The tol2 transposon system

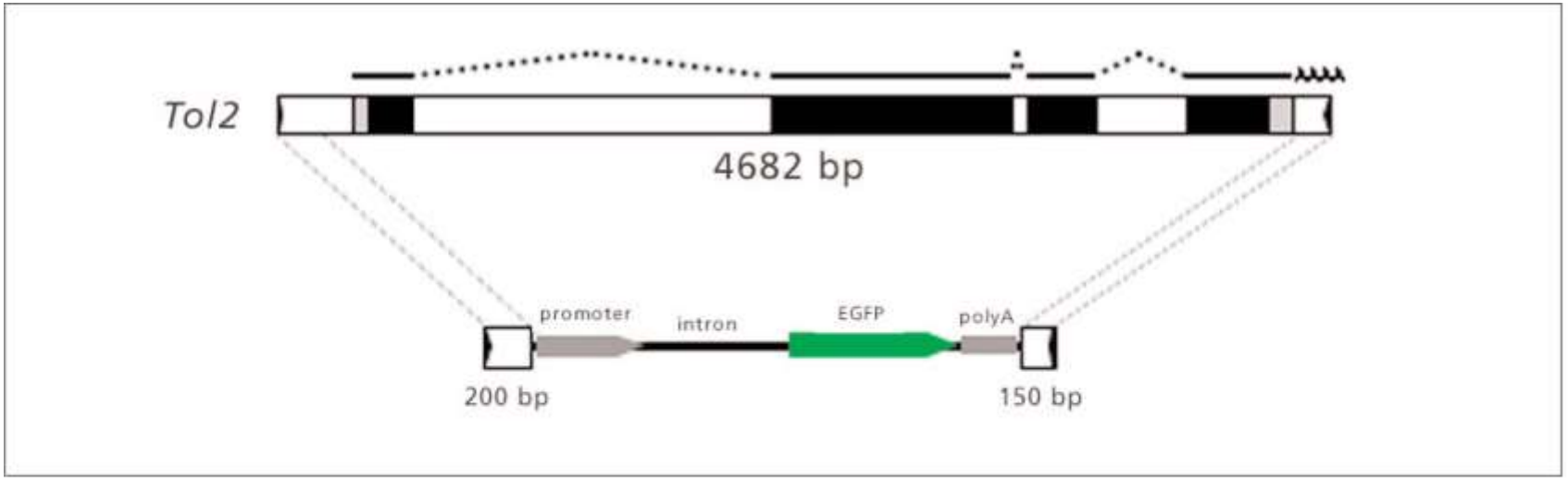
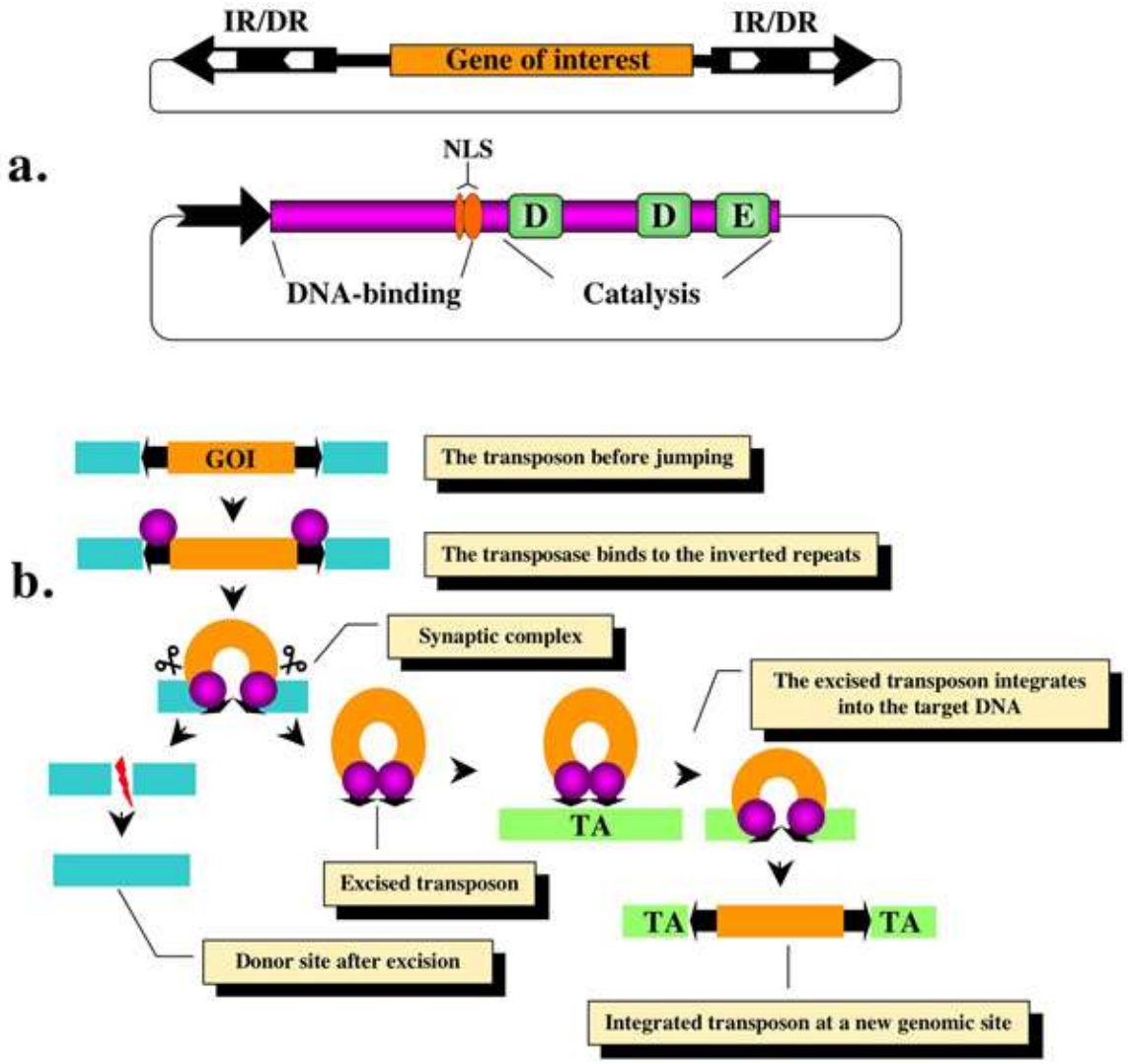
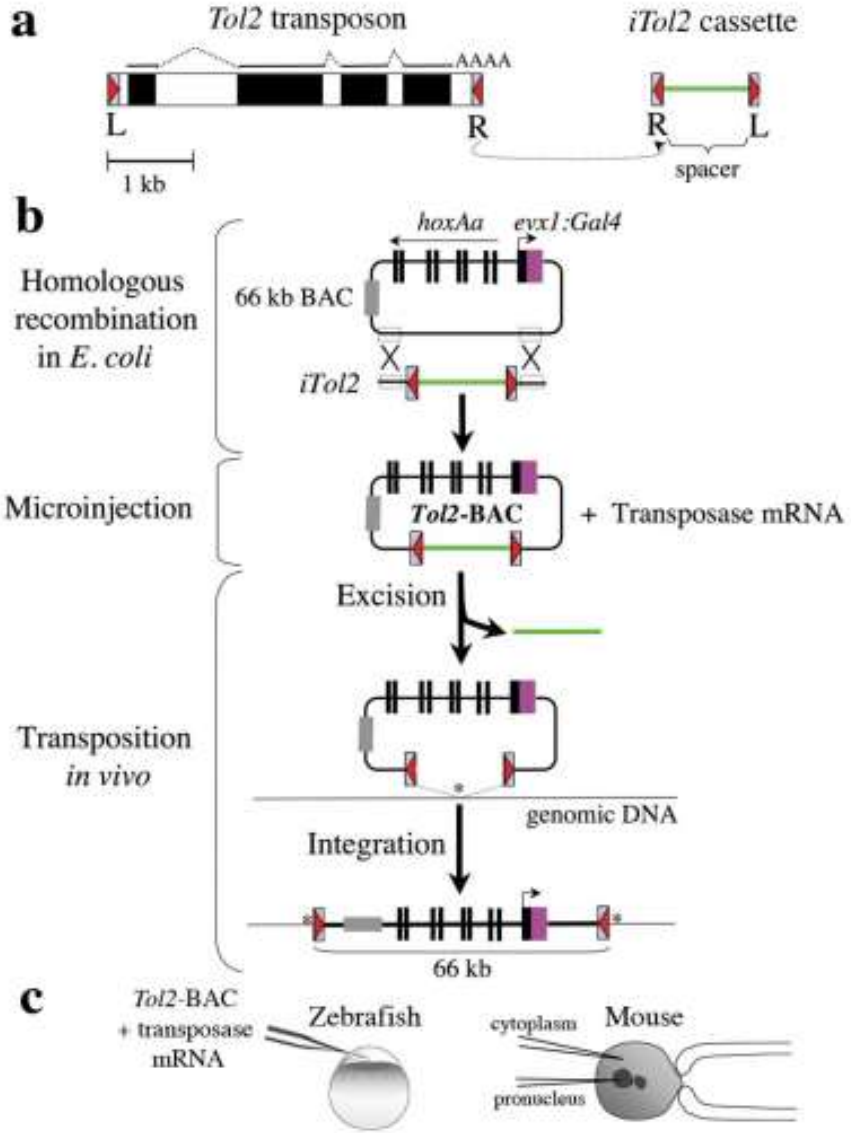


Figure 1
The structures of the *Tol2* transposable element and the minimal *Tol2* vector. At the top of the illustration is the 4,682 base pair (bp), full-length *Tol2*. RNA transcribed from *Tol2* that encodes a transposase protein [3] is shown by lines (exons) and dotted lines (introns). Black boxes and gray boxes represent coding regions and untranslated regions, respectively. Black arrowheads in boxes at both ends indicate 12 bp terminal inverted repeats (TIRs). The lower portion of the figure shows the minimal *Tol2* vector with the green fluorescent protein (GFP) expression cassette. The minimal *Tol2* vector contains 200 and 150 bp of DNA from the left and right ends, which include TIRs (black arrows) and subterminal regions (open boxes) [5]. The transposon vector can carry a DNA fragment, for example the GFP expression cassette in this figure, between these sequences.

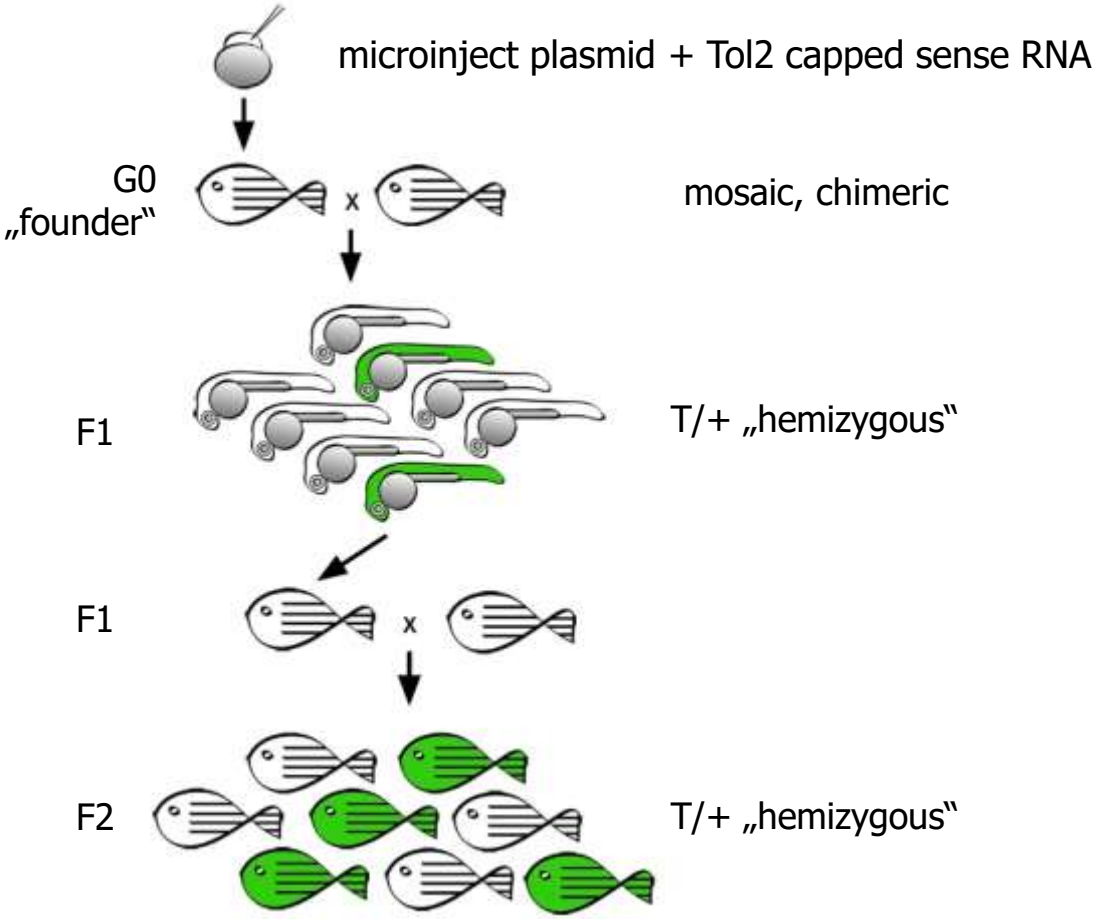
The Tol2 transposon system



BAC transgenesis



Transgenesis in zebrafish



Zebrafish as model system

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representative of its animal group	good
relevance for humans	good
many progeny	excellent
progeny all year round	excellent
fast generation time	OK
easy to house/culture	good
small (but not too small)	good
cheap	good
fast embryonic development	excellent
external development	excellent
transparency (imaging!)	excellent
accessible for embryological techniques (transplantations)	good
diploid (or haploid): ability to identify mutants	good
small genome	OK
inbred lines	poor
forward genetics	excellent
reverse genetics	excellent
RNAi	NO
transgenesis	excellent
pluripotent stem cell culture (ES cells)	NO
knock-ins/outs	in development

Housing in the lab

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- re-circulating freshwater systems (5-10% water exchange per day)
- reverse osmosis water (RO) + defined amount of salts
  OR: mixture of tap water (in Germany!) + RO water + salts

Water parameters

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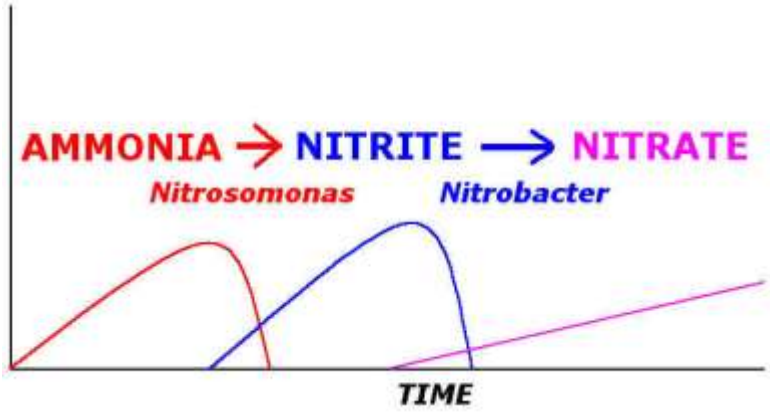
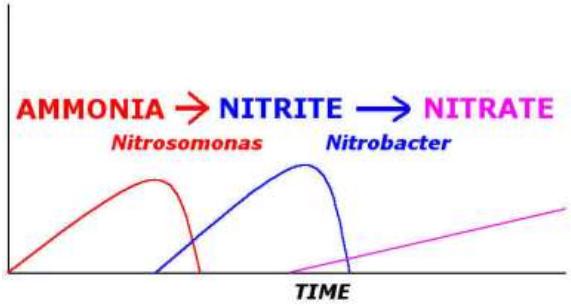
Parameter	Represents	Target	Comments	Controlled by
Conductivity	Total ion concentration	200 – 1000 µS Weidinger: 350µS (Siemens)	Higher might help to reduce energetic cost (osmotic balance); but not good to use salts that contain a lot of NaCl	Addition of $CaCO_3$ & $MgCO_3$ + trace elements And/or „Red Sea Salt", „Instant Ocean" or the like
Total hardness (GH)	All multivalent ions, particular Ca^{2+} and Mg^{2+}	5 ° dH	Too little causes bone and other defects	
Carbonate (temporary) hardness (KH)	Bicarbonate (HCO_3^-)	3 ° dH	Too much causes limescale	
Copper		0	Is toxic	No use of copper pipes!
Phosphate (PO_4)		< 5 mg/l	Fish don't care much, but high concentrations favor algae growth	Amount and type of food.
pH		7 - 8 Weidinger: 7.3		Sodium bicarbonate (usually pH needs to be brought up)
Temperature		24 – 30 °C (28.5°C) Weidinger: 27°C	Compromise between fast growth and bearable climate in room.	Air temperature needs to be similar to minimize evaporation.
Oxygen		At saturation: 7.8 mg/l at 28°C		Water flow, recirculation of water in storage tank

Nitrogen cycle



N excreted by fish (urine, feces) ends up in the water. Needs to be dealt with. Bacteria in the filters metabolize it.

Nitrogen cycle



Formula	English	Deutsch	relevance	Target	Comment
NH_3	Ammonia	Ammoniak	Very toxic	Cannot be measured	Should not accumulate, since it converts to NH_4^+ But: at high pH it might accumulate!
NH_4^+	Ammonium	Ammonium	harmless	< 0.02 mg/l	Harmless, but should not accumulate if bacteria-mediated nitrogen cycle works
NO_2	Nitrite	Nitrit	Toxic	< 10 mg/l	
NO_3	Nitrate	Nitrat	Rel. harmless	< 50 mg/l	Remove by water exchange

Disease: Mycobacterium

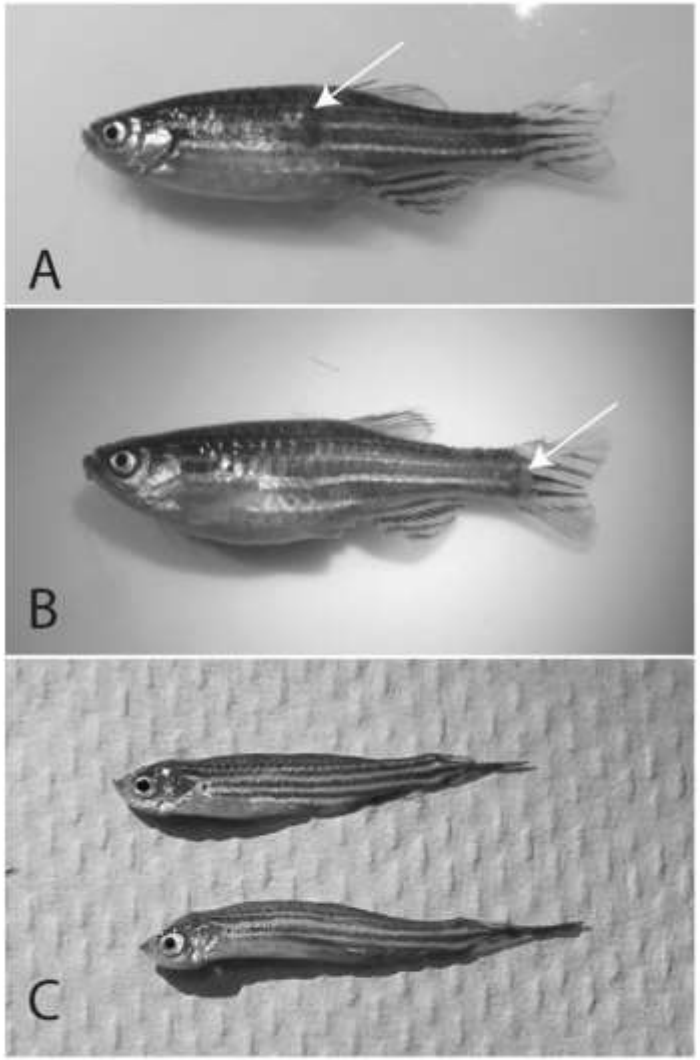


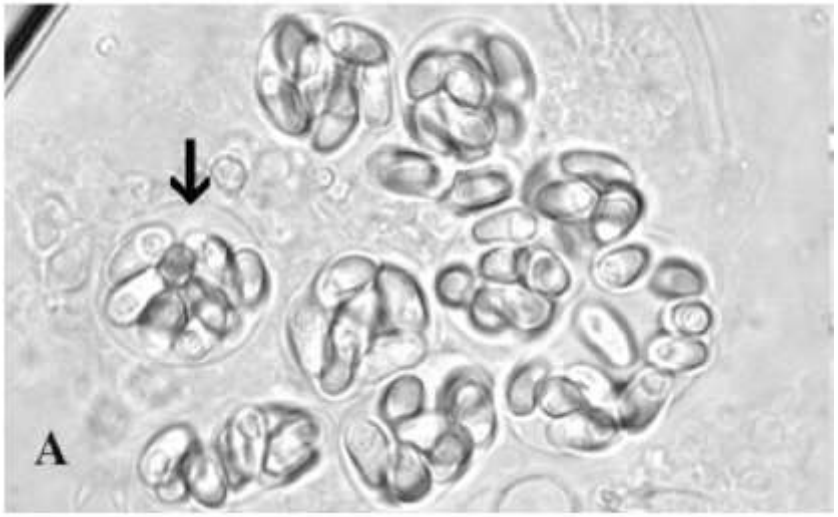
Figure 1 (**A, B**) External lesions (arrows) associated with *Mycobacterium marinum* infection in zebrafish. (**C**) Severe emaciation associated with *Mycobacterium haemophilum* infection.

Table 1 ***Mycobacterium*** **species known to infect zebrafish in research facilities**

Species	Source
Mycobacterium abscessus	Astrofsky et al. (2000); Watral and Kent (2007)
Mycobacterium chelonae	Astrofsky et al. (2000); Kent et al. (2004); Whipps et al. (2008)
Mycobacterium chelonae–like	Kent et al. (2004); Whipps et al. (2007a)
Mycobacterium fortuitum	Astrofsky et al. (2000)
Mycobacterium haemophilum	Whipps et al. (2007b)
Mycobacterium marinum	Watral and Kent (2007)
Mycobacterium peregrinum	Kent et al. (2004)

Whipps et al. (2012), ILAR 53, 85-105.

Disease: microsporidiosis



Sanders et al. (2012), ILAR Journal 52, 106.

intracellular parasites

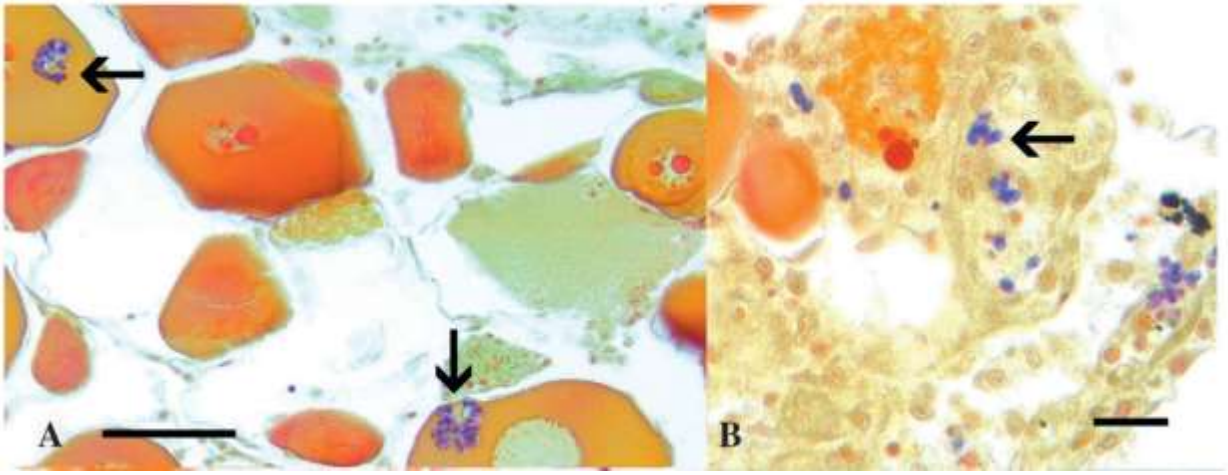
most prominent: *Pseudoloma neurophilia*

infects muscle, central nervous system, ovaries

reduced growth, emanciation, spinal deformation

often sub-clinical

detected in 75% of all fish facilities



gram-positive spores in follicles and stroma of ovary

Sanders et al. (2012), ILAR Journal 52, 106.

Hygiene

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Recirculating water is **filtered** and **sterilized**

- debris is allowed to settle in sump
- water is coarsely filtered through filter mats (which also contain bacteria)
- water is fine filtered in pressurized filters
- water is UV sterilized

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Cleanliness

1. feces and left-over food is removed from bottom of tanks
2. tank surfaces and lids are kept clean
3. removable tanks are washed (dishwasher) regularily
4. NO plants (real or artificial) are used
5. snails can be used to manage algae

Health management

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Monitoring

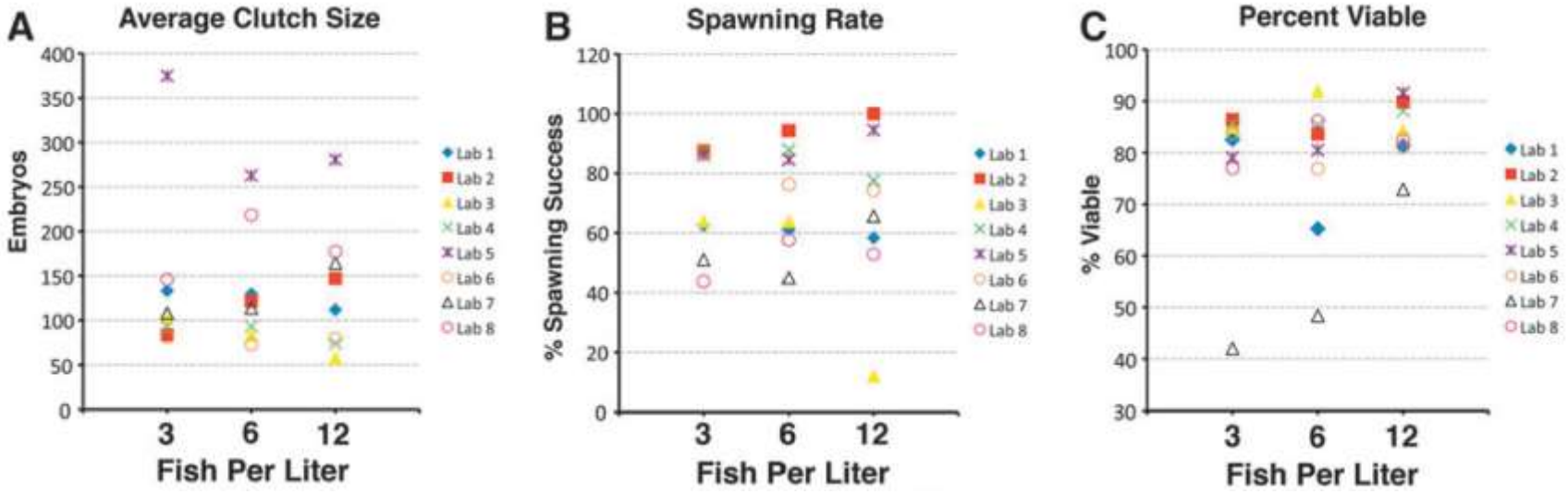
- fish status is monitored 2x daily
- dead or sick fish are removed (euthanised) immediately
- sick fish can be submitted to pathology services (ZIRC, Oregon)

Precautions

- **stress is kept at a minimum** (water parameters, nutrition, density)
- fish are never imported into main facility > quarantene > only embryos are transfered
- old fish are euthanized (> 2 years)
- embryos are bleached to remove parasites from chorions (which does not help against microsporidiosis)
- no street shoes and/or disinfection mats

Stocking density

- Zebrafish are social, form shoals > don‘t like to be kept individually for long periods of time
- Zebrafish diplay social order, in particular males. 2 males kept together will fight > avoid that, rather keep individually or in larger groups.



Castranova et al., Zebrafish 2011.

- Fish well-being as measured by reproductive success is not adversely affected by high stocking density (12 fish per liter).
  Weidinger: Usually 5/liter, permit to use 7/liter.

Food

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- In the wild: omnivorous. Zooplankton, insects (mostly aquatic, but also from surface), phytoplankton, algae, plant material...

- Fish dry food flakes (eg. Tetramin)
  must be refrigerated and administered dry.
- Live food: artemia brine shrimp.
  purchased as cysts. Hatch within 48h in aerated high salt water.
- Adults: 1-3 times a day. Can easily survive for 7 days without food.

Breeding

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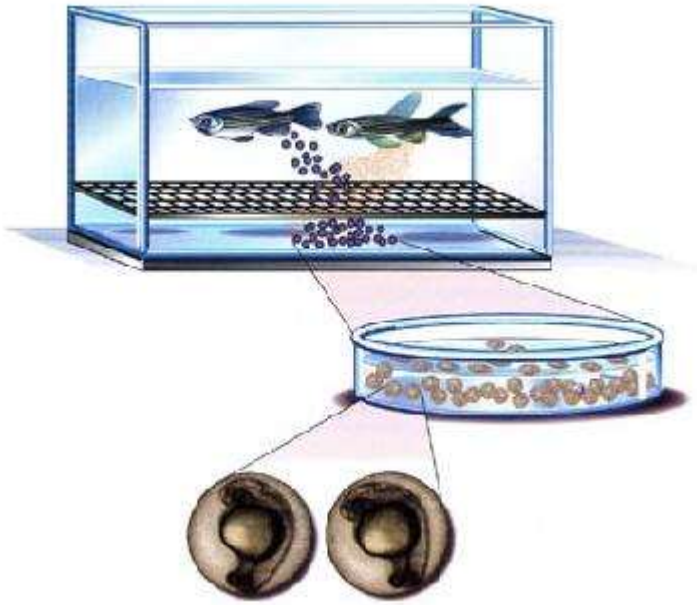
- day-night cycle (12-14 h day, 10-12 h night)
- fish spawn in the morning (till noon)
- male + female must spend the night together
- rel. small space (1 l per pair)
- they eat their progeny!

variations:

- timed egg lay via separation of male + female
- mass-spawning: eg. 4 males + 4 females
- in vitro fertilization



Carlos Manuel Díaz

Larval rearing

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- Embryos/larvae survive on yolk for 5 days.
- Day 5 – 12: Continuous food supply is best, no or very slow water flow.
- Different foods based on size of larvae:
  paramecia (easily cultured protozoa) OR rotifers (Protostomia)
  dry foods of increasing grain size, as often as possible

Growth rate depends heavily on nutrition and stocking density.
(Weidinger: 10 larvae/liter)

Sexual maturity can be reached within 5 weeks, usually within 2.5 months.

Resources

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Zebrafish International Resource Center (ZIRC), University of Oregon

protocols for husbandry, pathology services, source for wild-type and transgenic / mutant fish lines

European Zebrafish Resource Center, Karlsruhe Institute for Technology, ezrc.kit.edu

European repository for fish lines, screening facility

Zebrafish model organism database (ZFIN). zfin.org

Info on fish lines (transgenic, mutant), research reagents (antibodies, morpholinos), genome annotation

Zebrafish husbandry organisation. zhaonline.org

Non-profit, promotes husbandry standards through education & research

European Society for Fish Models in Biology and Medicine (EuFishBioMed)

promotes collaboration and exchange between fish labs