Zebrafish Procedures and Techniques

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7th Annual International Zebrafish Husbandry Course
Buguggiate, Italy 2018

Outline

- Squeezing males
- Squeezing females
- IVF –Fresh Samples
- Cryopreservation of Sperm
- Fin Clipping
- Swab DNA collection for Genotyping
- Microinjection
- Surface Sanitation of Embryos
  - Bleach
  - Bleach + PVP-1
- Histology dissection and fixation
- Shipping Fish
- Receiving Fish

In vitro Fertilization - Overview

The process of in vitro fertilization of zebrafish requires learning proficiency of two different techniques requiring the physical manipulation of fish. In addition, proper combination of the samples is important in achieving optimal fertilization.

Basic Process:

- Condition fish
- Extract sperm into glass capillary tube
- Normalize w/extender (~3:1 ratio of sperm to extender)
  - -Hank's Solution
  - -HBSS
  - -E400
- Store in tubes on ice until used for fertilization
- Extract embryos
- Add sperm w/extender on ice to clutch of embryos
- Activate w/water

Squeezing Males - Supplies

Materials Needed:

- **Anesthesia supplies and protocol**
- 35 mm petri dishes w/foam fish holder
- Kimwipes
- Millipore smooth forceps
- Glass capillary tubes
- Mouth pipette (or pipetman)
- Micro tubes or cryovials
- Ice bucket
- Conditioned male fish

Tricaine-S (MS222)

~4mls 3X buffered tricaine per 100mls fish water

Shallow container w/ fish water
-spawning baskets work well

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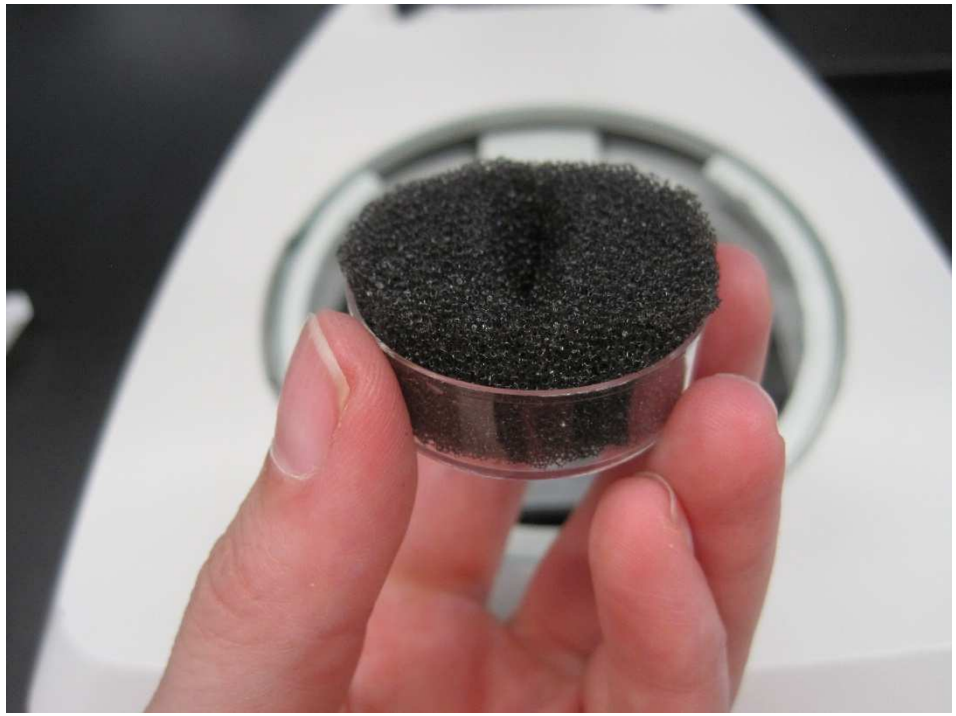

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Mouth pipettes are not allowed in some countries and at some biosecurity levels!!

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Separated from female fish prior to procedure

Some procedures call for:
-separation duration from 1 day up to 2 weeks
-rack or static housing in separate tanks
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Reasons for difference: strain, age of fish, environmental condition, available supplies

Squeezing Males - Supplies

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- Glass capillary tubes
- Mouth pipette (or pipetman)
- Micro tubes or cryovials
- Ice bucket
- Conditioned male fish
- **Sperm Extender**

Sperm extender is a hypotonic solution designed to prevent sperm activation that is used to dilute, normalize, and house sperm prior to IVF or cryopreservation.

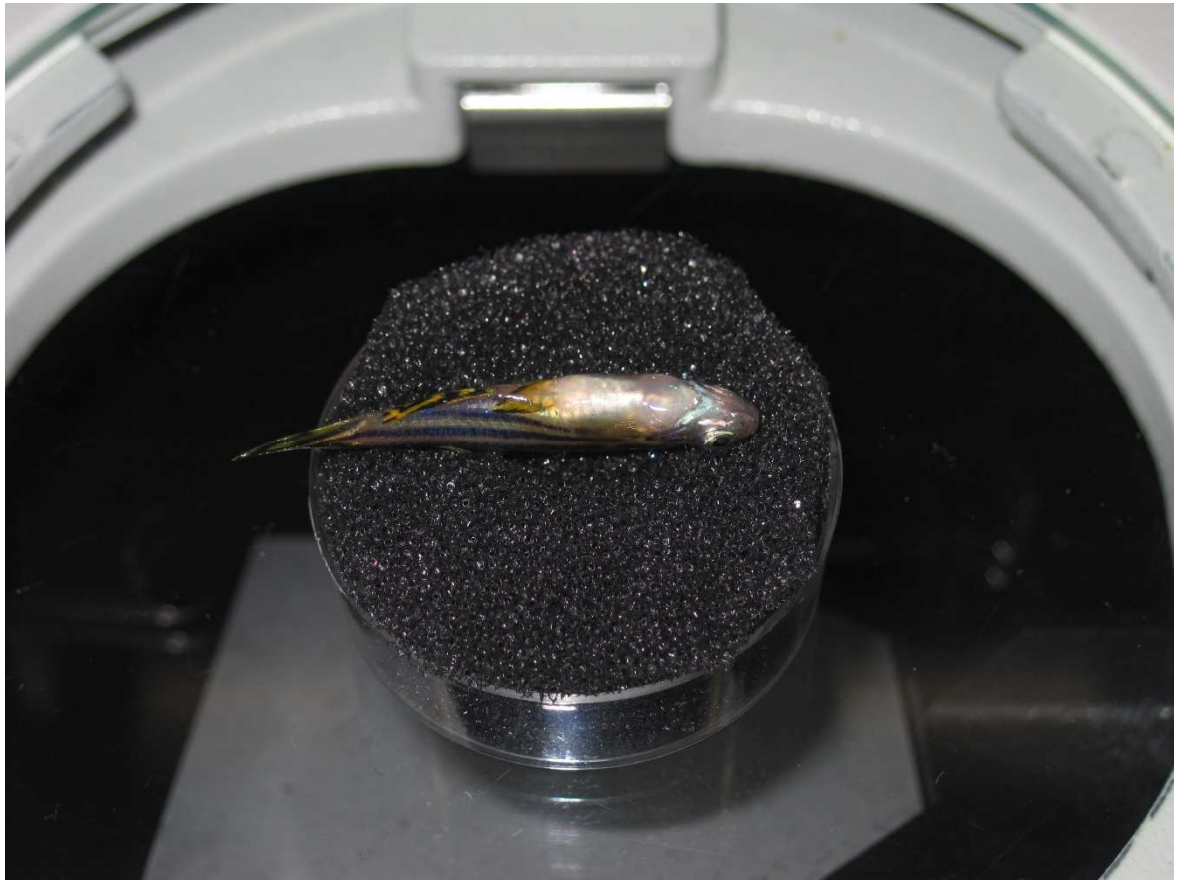
E400 (ZIRC)
https://zebrafish.org/documents/protocols/pdf/Cryopreservation_IVF/zirc_rmmb_freezing_protocol.pdf

Hank's Solution (Kimmel & Walker Method)
http://zfin.org/zf_info/zfbook/chapt2/2.8.html

HBSS (Sigma)
Commercially available pre made Hank's Solution

Squeezing Males - Procedure

1. Anesthetize fish per institutional protocol.
2. Place the fish, belly up into slit of foam holder.
3. Place the dish w/fish on the microscope stage and use top light for illumination.



Squeezing Males - Procedure

4. Gently wipe the ventral side of fish with the corner of the Kimwipe.
   -completely dry underside of fish as water activates sperm

5. Using the microcapillary, move the anal fins aside to expose the urogenital opening

6. Use the smooth forceps to gently squeeze side of fish
   -collect sperm using capillary action of the tube.

Squeezing Males - Procedure

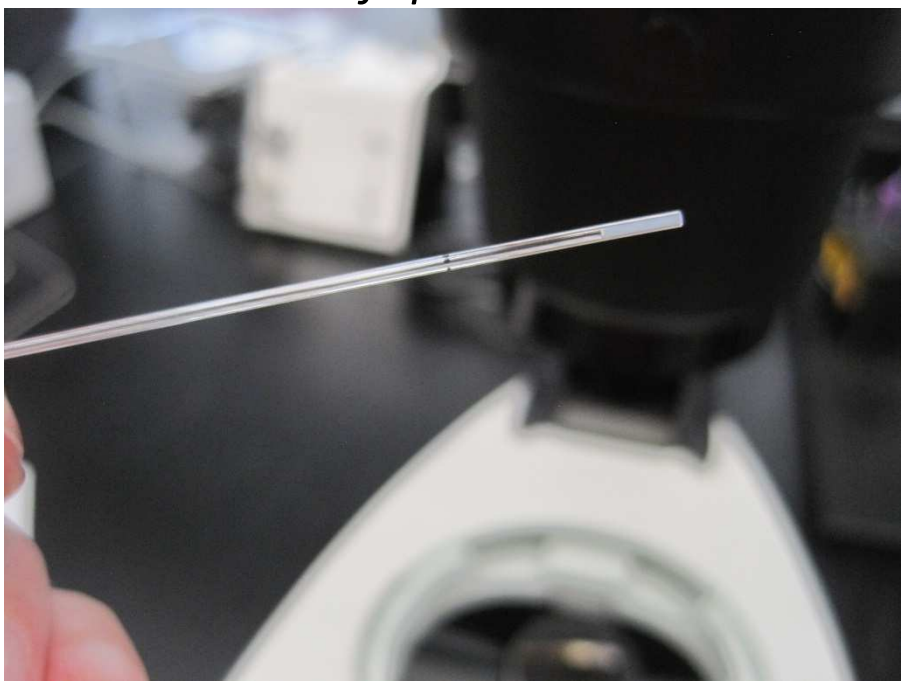
7. Return fish to recovery tank.

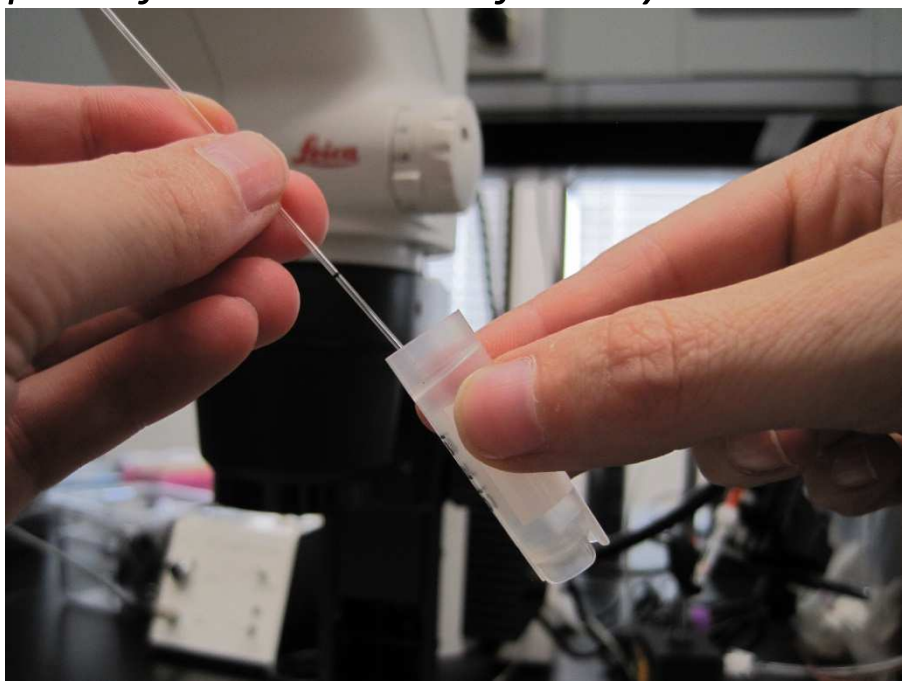
8. Normalize the sperm volume w/extender (~3:1 ratio of extender to sperm)

8. Expel the sample into an empty 0.6ml microtube

-samples on ice can be used for ~90 minutes post collection

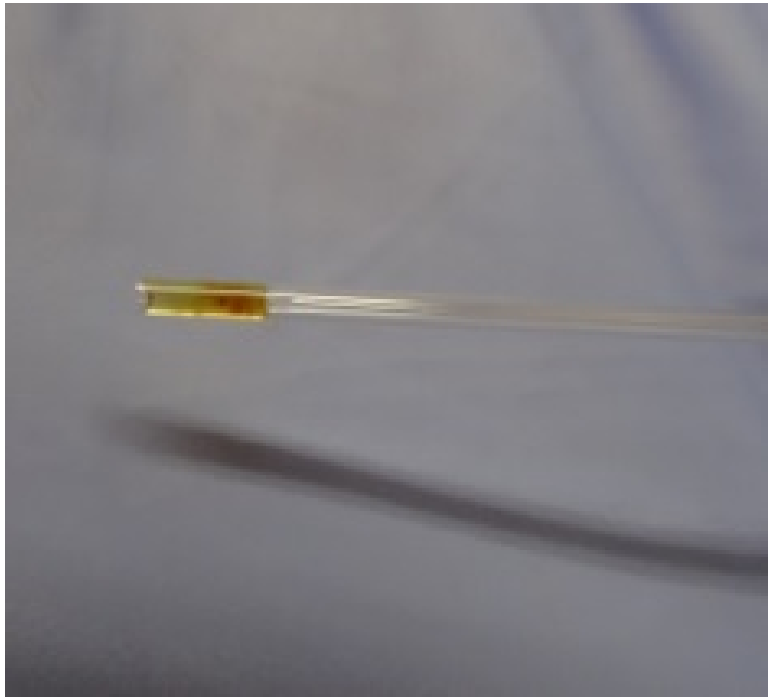
-~0.05 ml of sperm and extender is required for each clutch of embryos



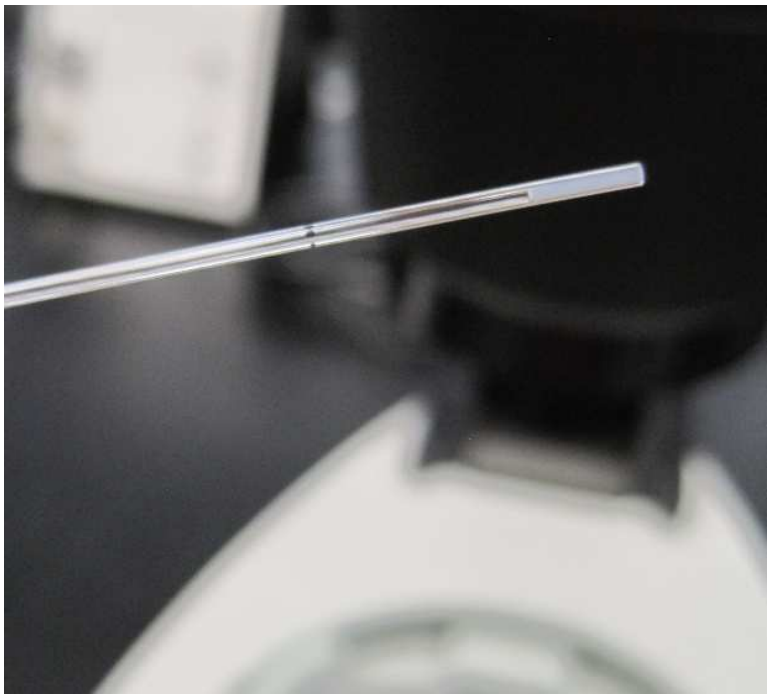


Squeezing Males – QA/QC

BAD! DO NOT USE!



GOOD!



Squeezing Females - Supplies

Materials Needed:

- **Anesthesia supplies & protocol**
- 35 mm petri dishes
- Metal spatula
- Preservation method
- Conditioned female fish

Tricaine-S (MS222)

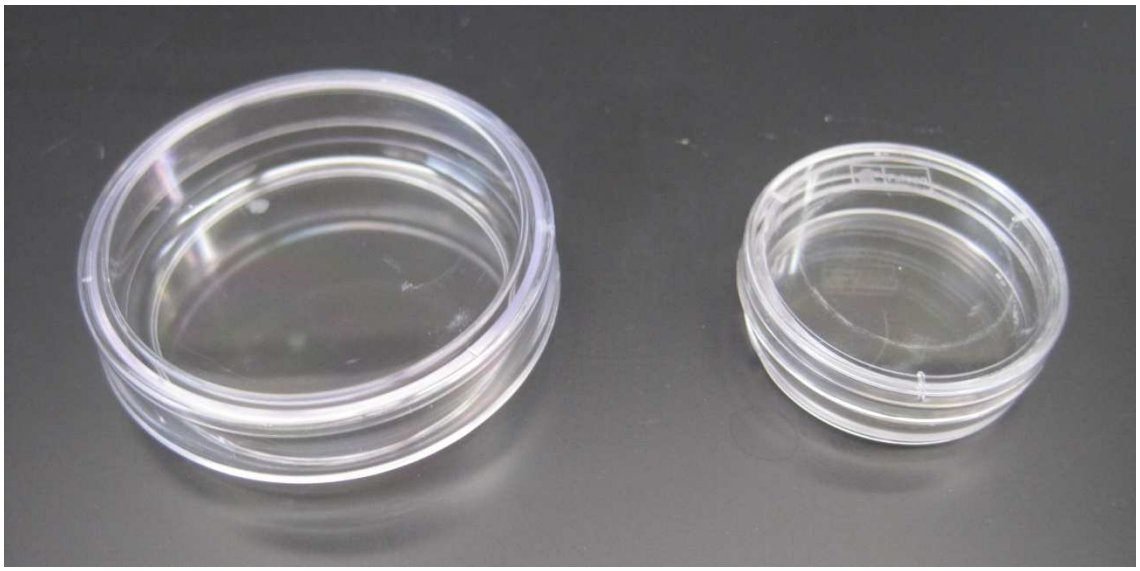
~4mls 3X buffered tricaine per 100mls fish water

Shallow container w/ fish water
-spawning baskets work well

Squeezing Females - Supplies

Materials Needed:

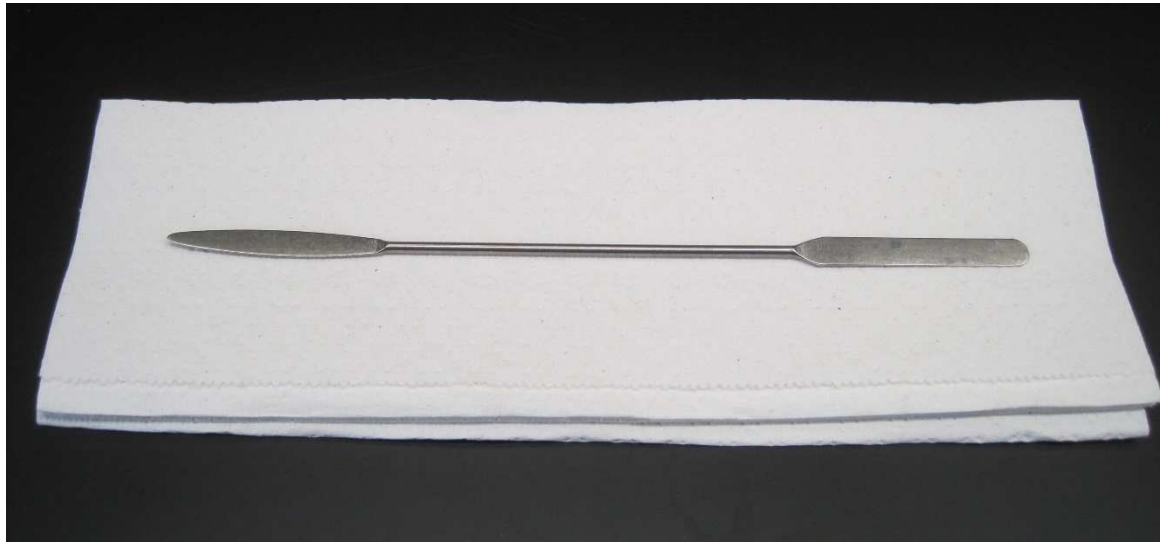
- Anesthesia supplies & protocol
- **35 mm petri dishes (or 60mm for larger fish**
- Metal spatula
- Preservation method
- Conditioned female fish



Squeezing Females - Supplies

Materials Needed:

- Anesthesia supplies & protocol
- 35 mm petri dishes
- **Metal spatula**
- Preservation method
- Conditioned female fish



Squeezing Females - Supplies

Materials Needed:

- Anesthesia supplies & protocol
- 35 mm petri dishes
- Metal spatula
- **Preservation method**
- Conditioned female fish

The need for a preservation method while embryos are on the bench awaiting fertilization is depended on a number of factors:

-Environmental condition of the work space
-Experimental goals

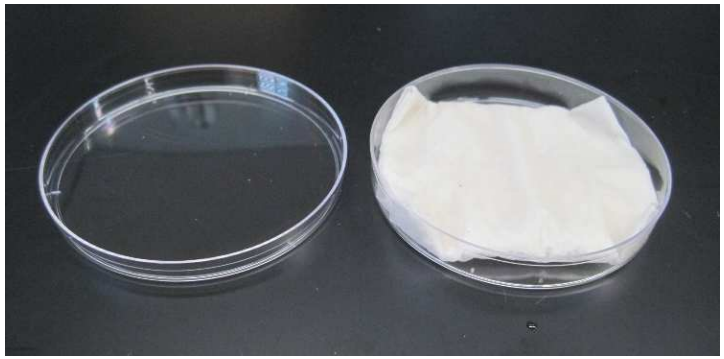
Squeezing Females - Supplies

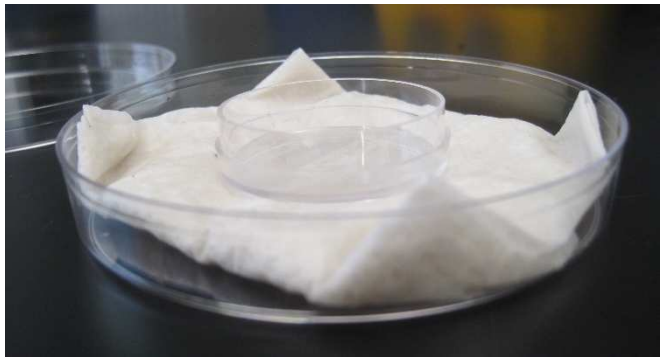
Materials Needed:

- Anesthesia supplies & protocol
- 35 mm petri dishes
- Metal spatula
- **Preservation method**
- Conditioned female fish

Humidity Chamber

- 150mm petri dish
- Paper towel nested in bottom
- Saturated in fish water
- 35mm petri dish (with eggs) nests inside
- Lid goes on while collecting embryo clutches





Squeezing Females - Supplies

Materials Needed:

- Anesthesia supplies & protocol
- 35 mm petri dishes
- Metal spatula
- **Preservation method**
- Conditioned female fish

Aquaboost OvaCoat - Cryogenetics

http://s13704.pcdn.co/wp-content/uploads/2014/04/OvaCoat.pdf

~100µl per clutch to extend bench life of embryos by ~30 minutes

Squeezing Females - Supplies

Materials Needed:

- Anesthesia supplies & protocol
- 35 mm petri dishes
- Metal spatula
- Humidity chamber
- **Conditioned female fish**

Separated from male fish prior to procedure

Some procedures call for:

-separation duration from 1 day up to 2 weeks
-rack or static housing in separate tanks
-males and females in same tank w/divider

Reasons for difference: strain, age of fish, environmental condition, available supplies

Squeezing Females - Procedure

1. Place female fish into anesthetic bath

2. Wait for gill movement to slow down and remove fish with the plastic spoon.

3. Dip the fish in a dish of clean fish water to remove anesthetic from exterior of fish

Squeezing Females - Procedure

4. Gently transfer fish from spoon to towel, roll once to dry surface.

5. Transfer fish from paper towel to 35 or 60 mm petri dish using the spatula

6. Slightly dampen your fingers with fish water

Squeezing Females - Procedure

7. Place index finger of non-dominant hand on the dorsal side of the fish.

8. Using index finger of the dominant hand, press gently from the belly (~mid trunk) towards the vent. If gentle pressure yields no embryos, do not continue to squeeze.

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

Squeezing Females - Procedure

10. Place the fish into a recovery tank. Wait until all fish are fully revived before putting them back on the system. (follow up observation later that day is suggested)

11. If pooling multiple clutches, utilize one of the preservation methods if needed

IVF & Cryopreservation

With a few additional supplies, the techniques of squeezing males and females can be used to execute:

- IVF w/fresh and frozen samples
- Cryopreservation

in *Vitro* Fertilization w/fresh samples

Order of operations:

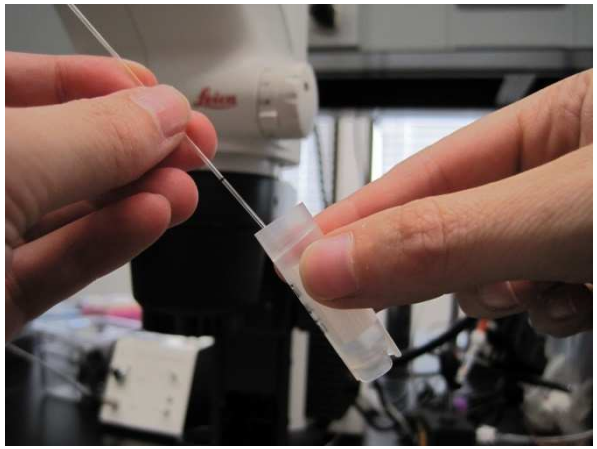
1. Separate fish using a condition methods prior to IVF event

2. 30 minutes prior to “dawn” set up stations for squeezing females and males

3. Squeeze male fish (using technique previously discussed)

4. Pool the sperm w/extender from several males or single males depending on need

-Sperm from 5-10 males is adequate for fertilization of several hundred eggs.
-Concentration of sperm can be estimated and should look like a cloudy suspension.



in *Vitro* Fertilization w/fresh samples

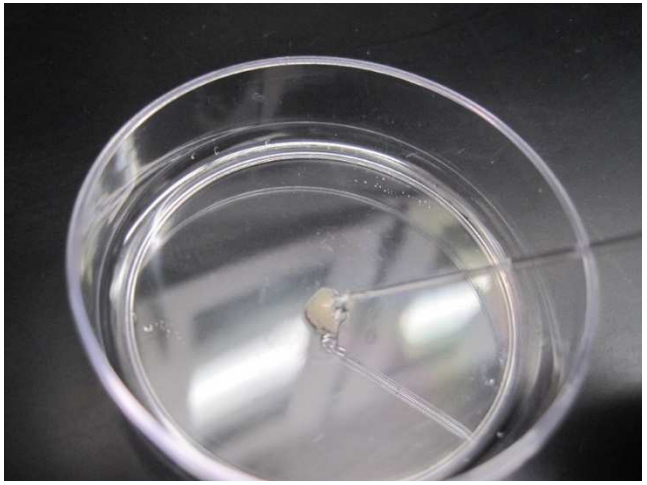
5. Begin squeezing female fish (pooled or individual depending on need)

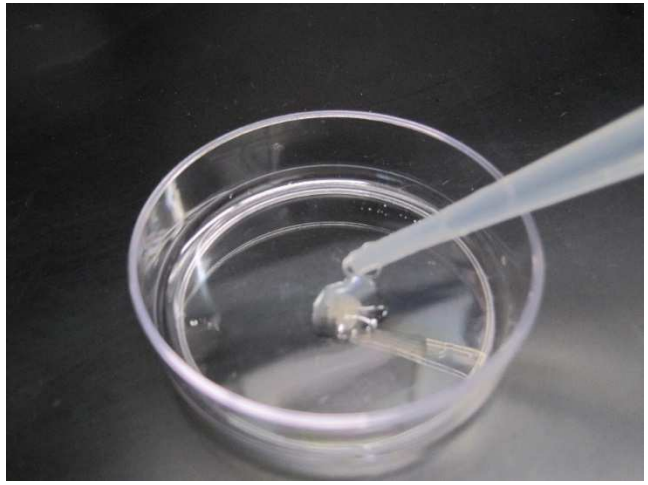
-use preservation method if eggs are not immediately being fertilized

6. Add 30-50µl of the sperm in extender directly over the clutch.

7. Mix gently with the capillary or pipette tip, and then add ~0.5ml of fish water.

8. Wait 1-2 minutes and add an additional 2ml fish water.





Cryopreservation

Cryopreservation is a valuable tool. Fish lines and populations can be preserved for future use, rederivation, or to re introduce genetic vigor. The protocols available range from fairly simple to extremely complex.

Timing and proficiency at all aspects of the procedure are vital and often require dedicated personnel doing the process on a routine basis (weekly) to achieve best results with the more complicated protocols.

Cryopreservation - Protocols

New ZIRC Method - 2017

https://zebrafish.org/documents/protocols/pdf/Cryopreservation_IVF/zirc_rmmb_freezing_protocol.pdf

Old ZIRC Method -2009

https://zebrafish.org/documents/protocols/pdf/Cryopreservation_IVF/ZIRCCryo&%20IVF.pdf

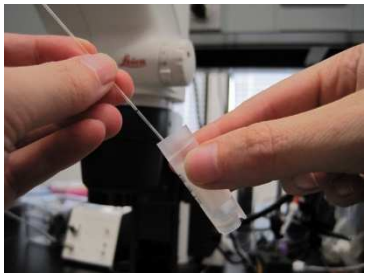
Draper/Moens Method - 2004

http://authors.fhcrc.org/323/2/JoVE_Protocol_1395.pdf

Cryopreservation

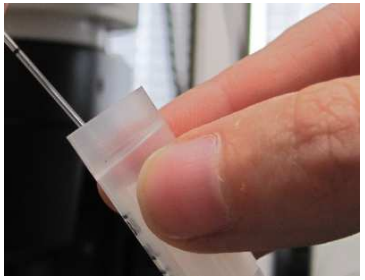
Milt is extracted from male fish

Normalized with an extender



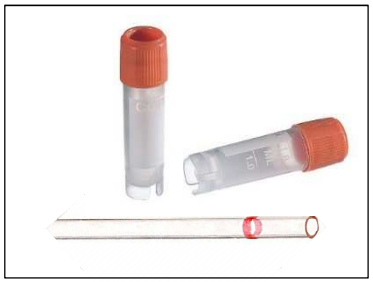
- Hank's Solution (www.zfin.org)
- HBSS (premade)
- E400 (ZIRC)
- Spermcoat (Cryogenetics)

Add cryoprotectant/ freeze medium



- Ginsberg Ringers (Moens/Draper)
- Raffinose Freezing Media - RMMB (ZIRC)

Sample Vessel



- Cryo vials
- Microcapillary tubes

Freeze

- Dry Ice
- Liquid Nitrogen

Storage

- Freezer
- Dewer

Cryopreservation

What procedure is right for you? This will depend on your goals:

Highest fertilization rate possible and the need to store fewer samples?

Production level needs

- investment in staff training, supplies and equipment
- dedicated space
- ability to do the procedure with regularity
- proficiency at making complex solutions

Average fertilization rate with allowance for more samples?

Basic preservation and rederivation of lines

- staff less regularly performing the procedure
- as long as you preserve the line are fertilization rates a concern?

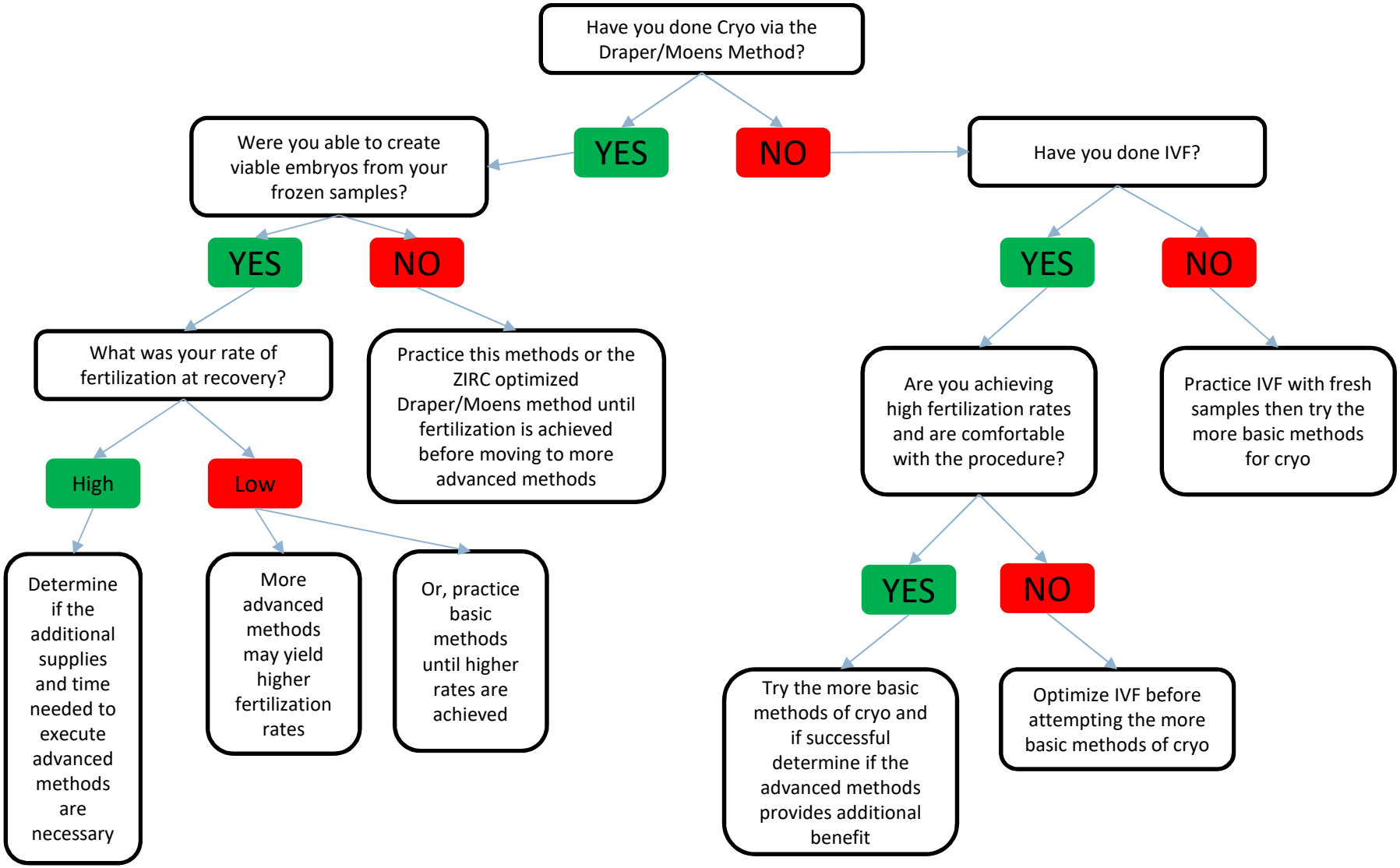
When is more cost effective to hire a contractor to do this procedure as needed?

Cost benefit analysis

- limited staff
- limited space
- limited resources

Cryopreservation

Decision tree based on familiarity with techniques



Genotyping Techniques

Husbandry staff are becoming increasingly more involved in the line maintenance process at facilities. Current methods of tissue collection for genotyping purposes are **fin clipping** and **skin swabbing**.

Fin Clipping - Supplies

Materials Needed:

- **Anesthesia supplies and protocol**
- Gated spawning baskets/# 1-88
- 96 well PCR plate
- Plate template for notes/results
- Several square sheets of Parafilm
- Scalpel or razor blade
- Sharp forceps
- Beaker of water and kimwipes
- Ice bucket

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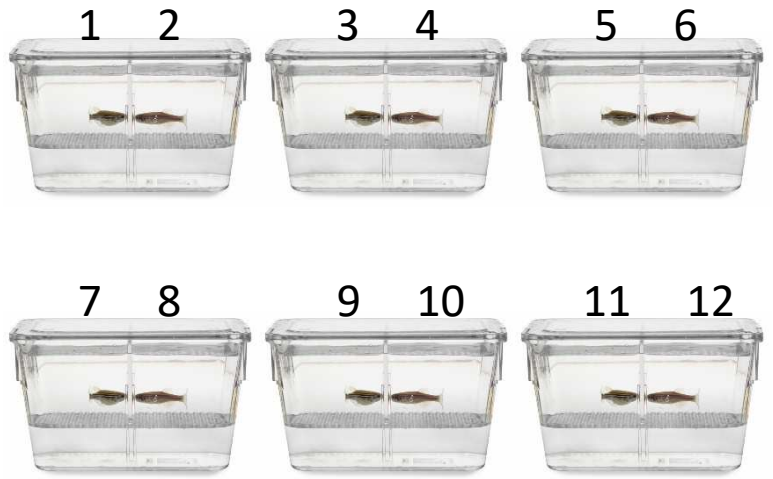

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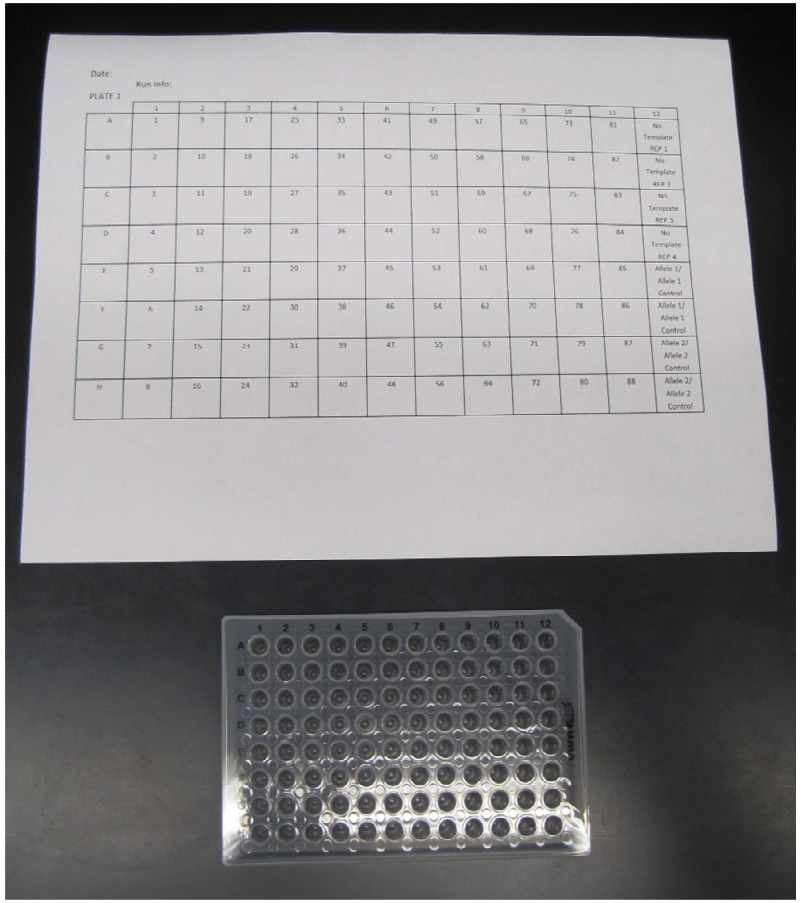

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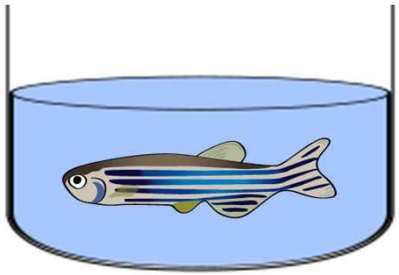
Fin Clipping - Procedure

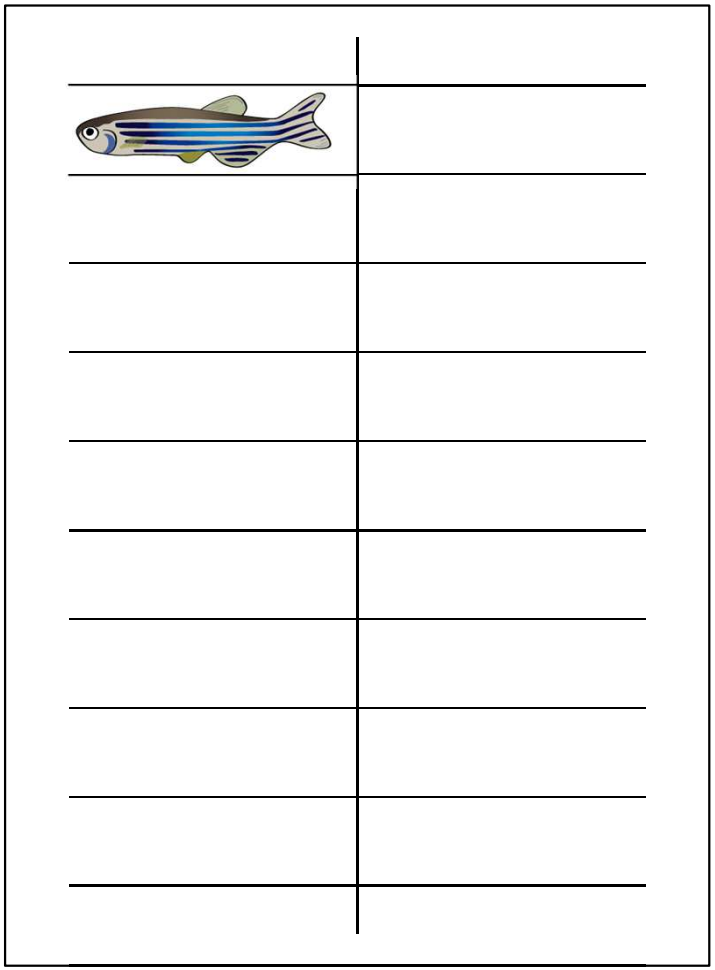
1. Set up clipping station, gated baskets and fish

2. Anesthetize fish.



3. Remove from anesthetic with spoon, remove excess water with paper towel

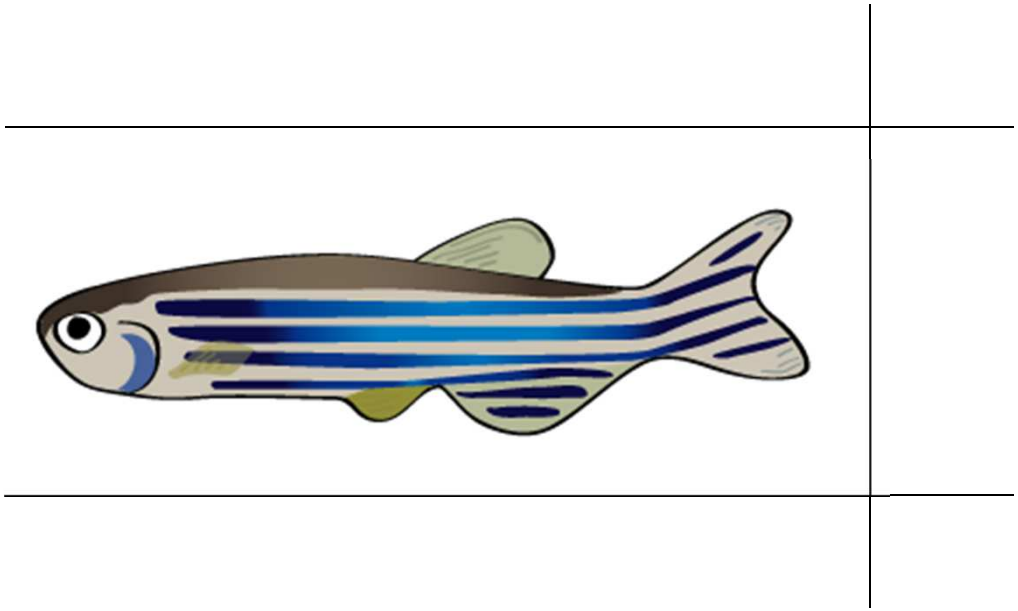
4. Transfer fish to top left spot on parafilm



Fin Clipping - Procedure

5. Firmly cut a portion of the tail off using the scalpel.

6. Use spoon to scoop fish up, and place in numbered spawning basket



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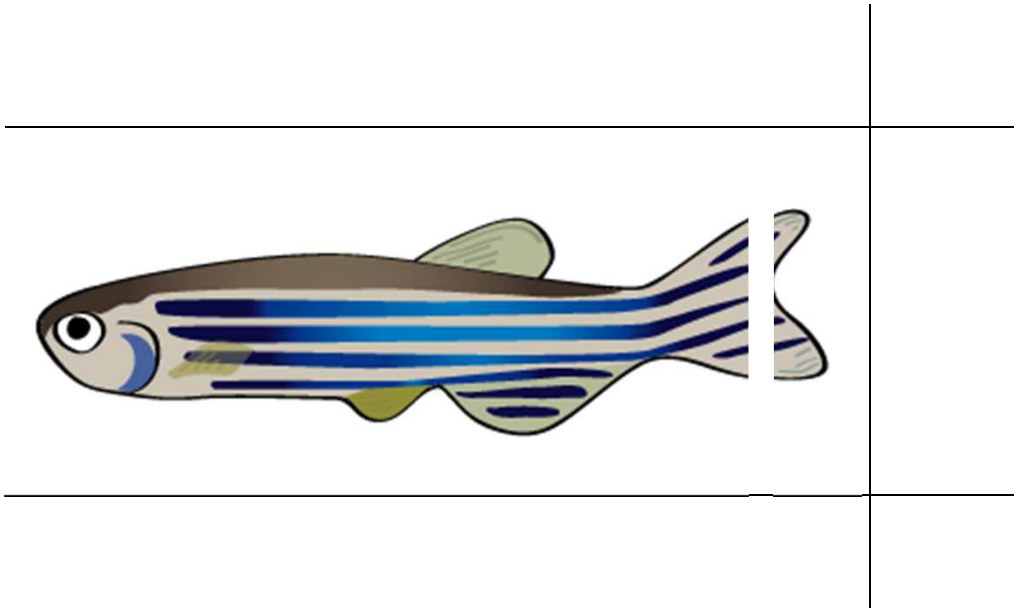


Fin Clipping - Procedure

7. Using forceps, pick up fin clip and place in corresponding well on plate
8. Dip forceps and scalpel in water, dry on kimwipe
9. Repeat this process, each time moving down to a fresh space on parafilm.

- Consistent clip sizes will make the PCR process easier
- You don’t need a huge piece! Leave a healthy portion of tail!!

Genotyping via Skin Swab

Materials Needed:

- **Sterile rayon tip swabs**
- **Wetted sponge**
- **Soft mesh net**
- **PCR protocol (see publication)**

ZEBRAFISH
Volume 13, Number 6, 2016
Mary Ann Liebert, Inc.
DOI: 10.1089/zeb.2016.1348

Original Article

A Low-Cost Method of Skin Swabbing for the Collection of DNA Samples from Small Laboratory Fish

Carl Breacker, Iain Barber, William H.J. Norton, Jonathan R. McDearmid, and Ceinwen A. Tilley

Abstract

Fin clipping of live fish under anesthesia is widely used to collect samples for DNA extraction. An alternative, potentially less invasive, approach involves obtaining samples by swabbing the skin of nonanesthetized fish. However, this method has yet to be widely adopted for use in laboratory studies in the biological and biomedical sciences. Here, we compare DNA samples from zebrafish *Danio rerio* and three-spined sticklebacks *Gasterosteus aculeatus* collected via fin clipping and skin swabbing techniques, and test a range of DNA extraction methods, including commercially available kits and a lower-cost, in-house method. We verify the

Breacker C, Barber I, Norton WHJ, McDearmid JR, Tilley CA. A Low-Cost Method of Skin Swabbing for the Collection of DNA Samples from Small Laboratory Fish. *Zebrafish*. 2017;14(1):35-41. doi:10.1089/zeb.2016.1348.

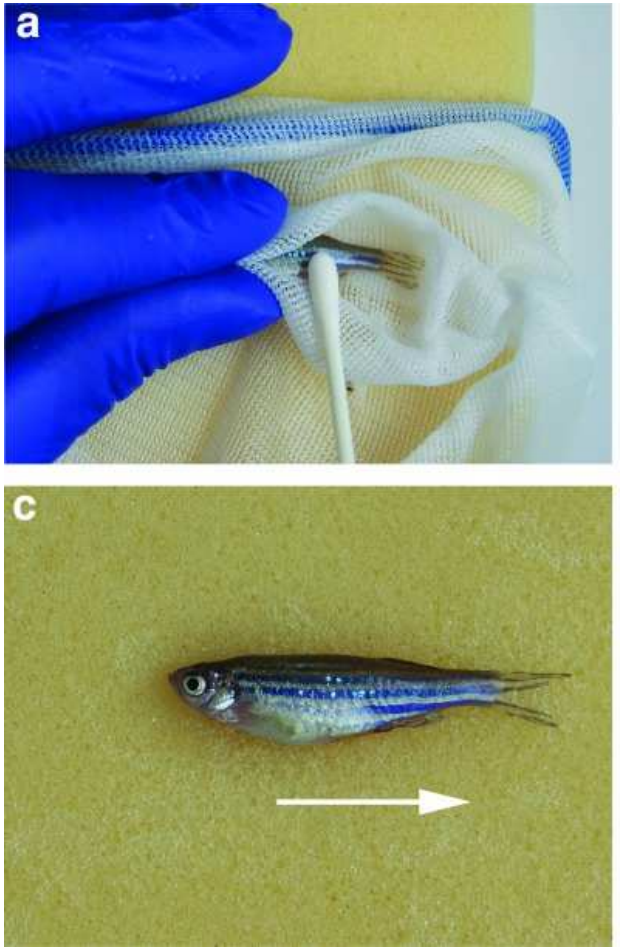
Genotyping via Skin Swab

1. Net fish and gently restrain over a wet sponge, restraining with net and forefinger and thumb.

2. Gently swab fish 5 times along flank from the operculum to the caudal fin

3. Return fish to individual housing tank (on system) while awaiting genotyping results

Direction Matters!! Always swab from head to tail!



Breacker, C. A Low-Cost Method of Skin Swabbing for the Collection of DNA Samples from Small Laboratory Fish. *Zebrafish*.

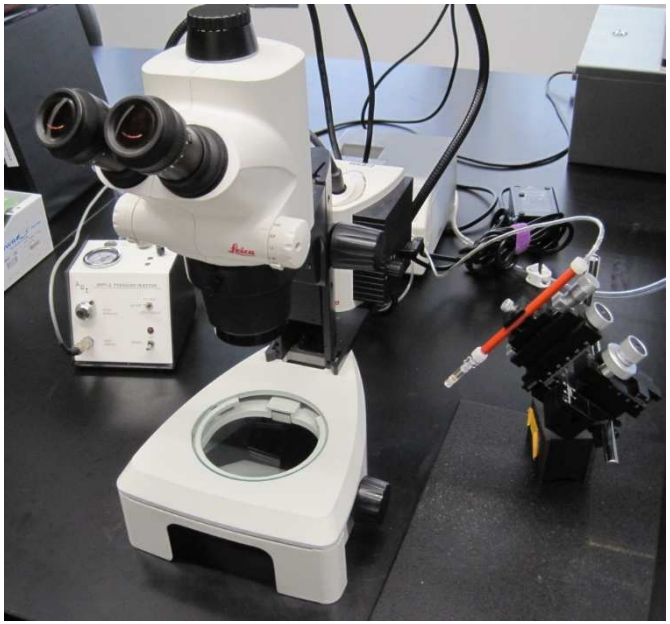
Laboratory Techniques

- Microinjections
- Dissection and fixation of whole fish for histological analysis

Microinjection - Supplies

Materials Needed:

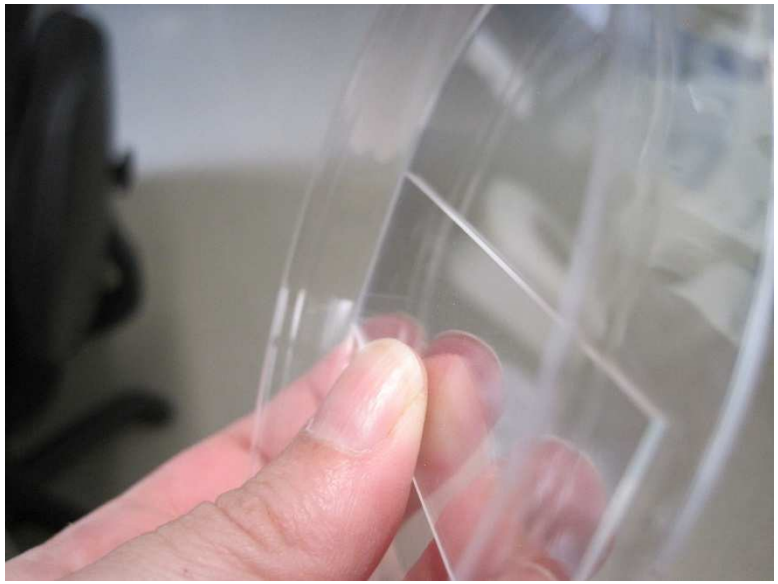
- **Microscope**
- **Micromanipulator**
- **Pressure injector/air tanks**
- 100mm petri dishes
- Glass microscope slide
- Agar injection molds
- Pipette
- Fish in gated crosses or iSpawn
- Injection needles
- Compound – Vector - Dye



Microinjection - Supplies

Materials Needed:

- Microscope
- Micromanipulator
- Pressure injector/air tanks
- **100mm petri dishes**
- **Glass microscope slide**
- Agar injection molds
- Pipette
- Fish in gated crosses or iSpawn
- Injection needles
- Compound – Vector - Dye



Glass slide methods is ideal for injections into the yolk stream

- Morpholinos
- Emulsions
- Dyes

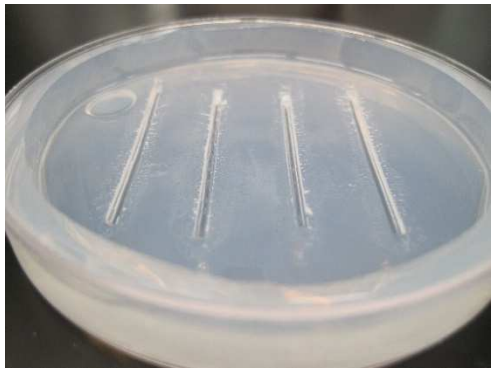
Microinjection - Supplies

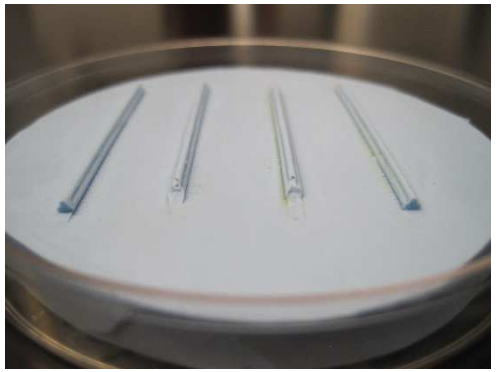
Materials Needed:

- Microscope
- Micromanipulator
- Pressure injector/air tanks
- 100mm petri dishes
- Glass microscope slide
- **Agar injection molds**
- Pipette
- Fish in gated crosses or iSpawn
- Injection needles
- Compound – Vector - Dye

Agar mold methods is ideal for injections into the single cell, which requires orienting the embryo more precisely

- **cDNA**
- **RNA**





Microinjection - Supplies

Materials Needed:

- Microscope
- Micromanipulator
- Pressure injector/air tanks
- 100mm petri dishes
- Glass microscope slide
- Agar injection molds
- **Pipette**
- Fish in gated crosses or iSpawn
- Injection needles
- Injection material - Dye

Brand: Kimble Chase
Product # is 63A53WT
Description: 5 ¾ borosilicate (Boro) glass Pasteur pipette

Microinjection - Supplies

Materials Needed:

- Microscope
- Micromanipulator
- Pressure injector/air tanks
- 100mm petri dishes
- Glass microscope slide
- Agar injection molds
- Pipette
- **Fish in gates spawning configuration**
- Injection needles
- Injection material- Dye

Microinjection - Supplies

Materials Needed:

- Microscope
- Micromanipulator
- Pressure injector/air tanks
- 100mm petri dishes
- Glass microscope slide
- Agar injection molds
- Pipette
- Fish in gated crosses or iSpawn
- **Injection needles**
- Injection material- Dye

1.0mm OD glass capillary w/filament
Micropipette puller
Or
Pre-pulled needles

Microinjection - Supplies

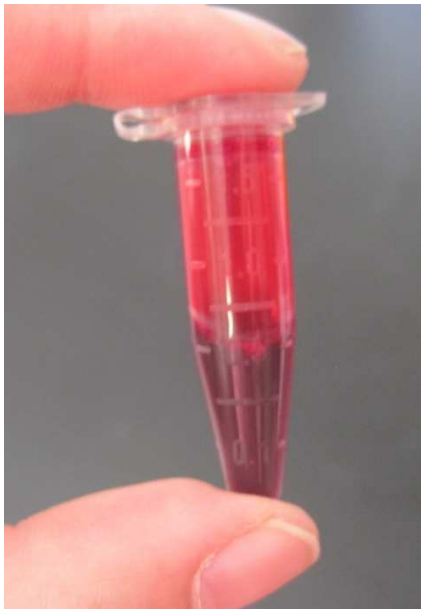
Materials Needed:

- Microscope
- Micromanipulator
- Pressure injector/air tanks
- 100mm petri dishes
- Glass microscope slide
- Agar injection molds
- Pipette
- Fish in gated crosses or iSpawn
- Injection needles
- **Injection material - Dye**

CRISPR/Cas9

CDNA, RNA

Morpholinos



Microinjection – Media & Volumes

Compound

+

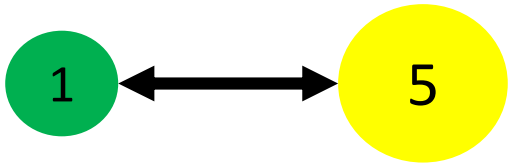
Sterile liquid

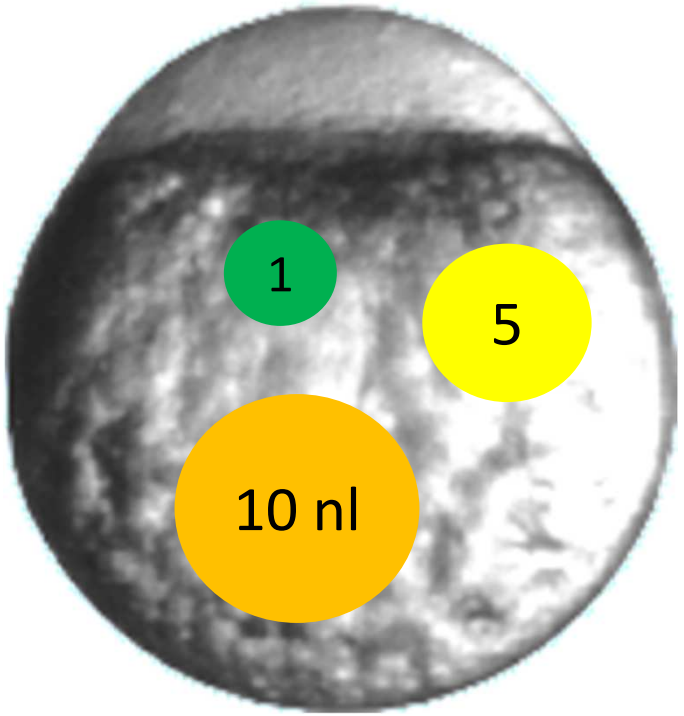
+

Tracer dye (phenol red)

=

Total injection volume





Microinjection – Media & Volumes

Compound

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Tracer dye (phenol red)

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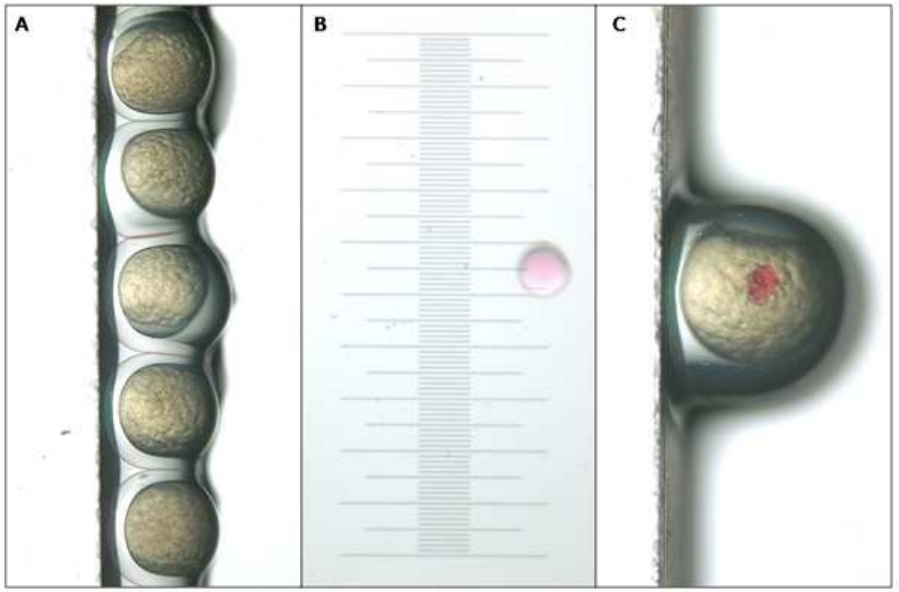
Total injection volume

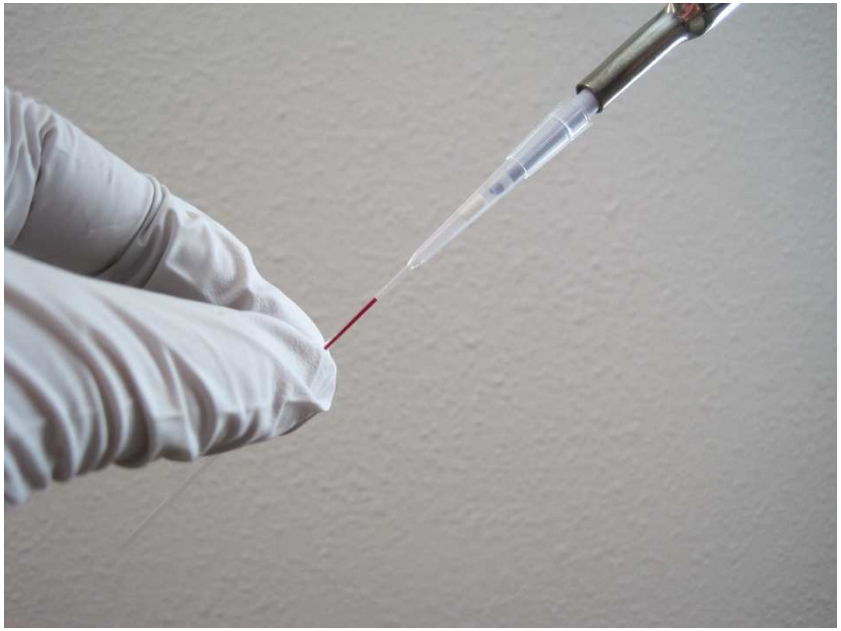


http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2762901/

- Place a drop of mineral oil on a micrometer
- Break tip of needle with forceps
- Injection into the oil
- a bead with a diameter of 0.1 mm contains 1nL

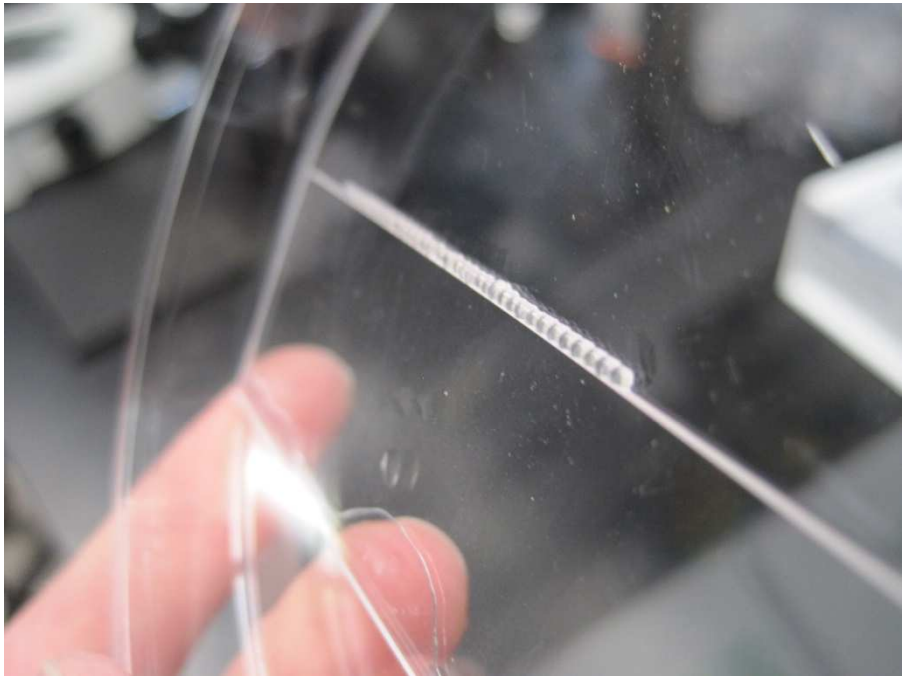
Microinjection - Procedure

1. Pull gates from spawning baskets or activate iSpawn
2. Wait ~15-20 minutes for fish to spawn, set up injection station during this time
3. Load needles with compound, and brake tip with fine forceps
4. Once fish are visibly producing embryos, collect with strainer



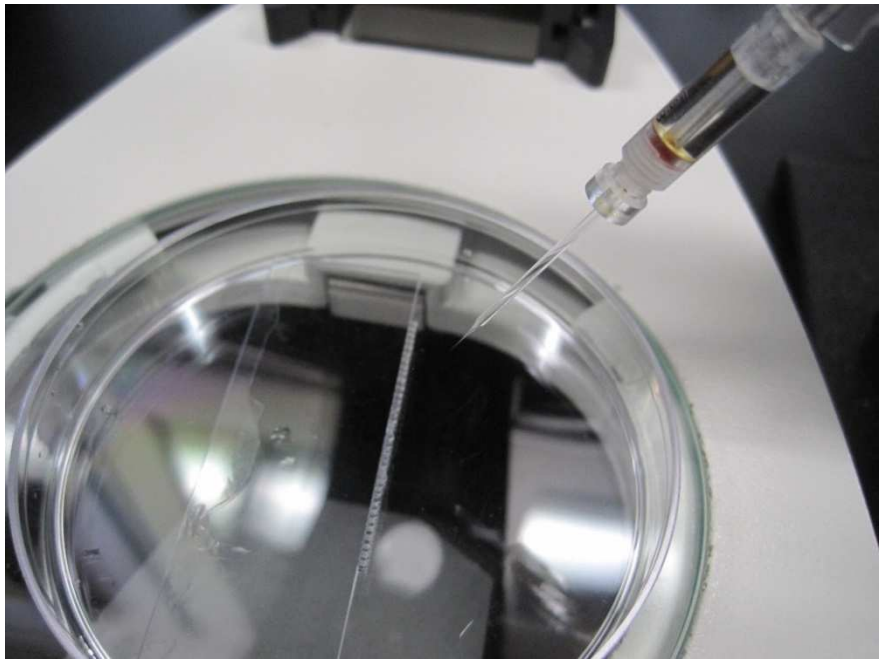
Microinjection - Procedure

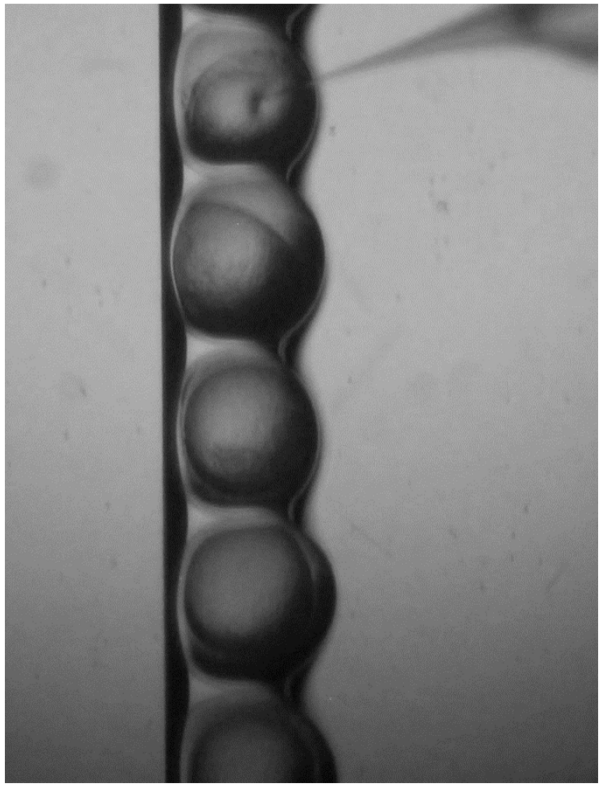
5. Select fertilized embryos with a glass pipette
6. Dispense embryos along the shelf created by the slide and petri dish
7. Place petri dish on microscope stage and begin injecting



Microinjection - Procedure

8. Lower the needle toward the top embryo
9. Pierce the surface of the chorion and enter the yolk in one motion
10. Depress injector pedal and smoothly retract the needle
11. Repeat down the column of embryos
12. Rinse embryos into petri dish w/embryo media





Dissection and Fixation for Histology

Materials Needed:

- **Euthanasia supplies and protocol**
- Live fish
- Razor blade or scalpel
- Micro dissecting scissors
- Forceps
- Fixative
- Rocker
- Vials or sealable tubes
- Parafilm

Tricaine-S (MS222)

~13 mls 3X buffered tricaine per 100mls fish water

Dissection and Fixation for Histology

Materials Needed:

- Euthanasia supplies and protocol
- **Live fish**
- Razor blade or scalpel
- Micro dissecting scissors
- Forceps
- Fixative
- Rocker
- Vials or sealable tubes
- Parafilm

http://zdmsociety.org/zebrafish-and-human-disease/

Fish must be freshly euthanized prior to fixation. Fish found dead are not appropriate samples for histology as the process of autolysis has already begun.

Dissection and Fixation for Histology

Materials Needed:

- Euthanasia supplies and protocol
- Live fish
- **Razor blade or scalpel**
- **Micro dissecting scissors**
- **Forceps**
- Fixative
- Rocker
- Vials or sealable tubes
- Parafilm

Dissection and Fixation for Histology

Materials Needed:

- Euthanasia supplies and protocol
- Live fish
- Razor blade or scalpel
- Micro dissecting scissors
- Forceps
- **Fixative**
- Rocker
- Vials or sealable tubes
- Parafilm

Dietrich's Solution

- 30 ml Ethanol (95%)
- 10ml Formalin

(Formaldehyde 37% solution, histological grade, contains 10-15% methanol)

- 2 ml Glacial Acetic Acid
- 58 ml Distilled water

- OR -

10% Formalin

This will likely depend on the service provider

Dissection and Fixation for Histology

Materials Needed:

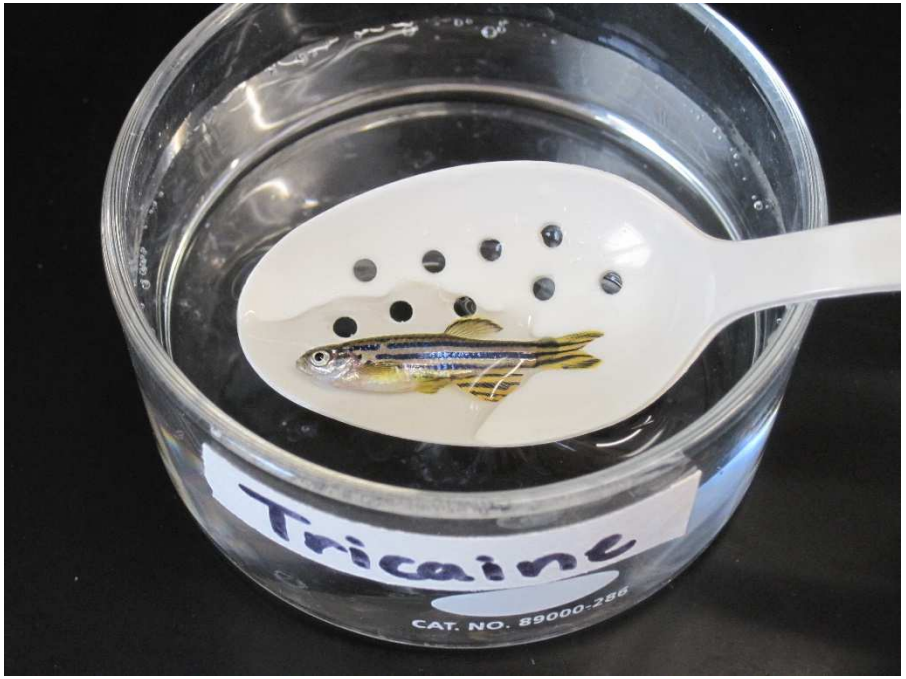
- Euthanasia supplies and protocol
- Live fish
- Razor blade or scalpel
- Micro dissecting scissors
- Forceps
- Fixative
- **Rocker**
- **Vials or sealable tubes**
- **Parafilm**

*cut into strips

Dissection and Fixation for Histology

1. Euthanatize fish using an overdose of MS-222 (Tricaine) or other approved method.

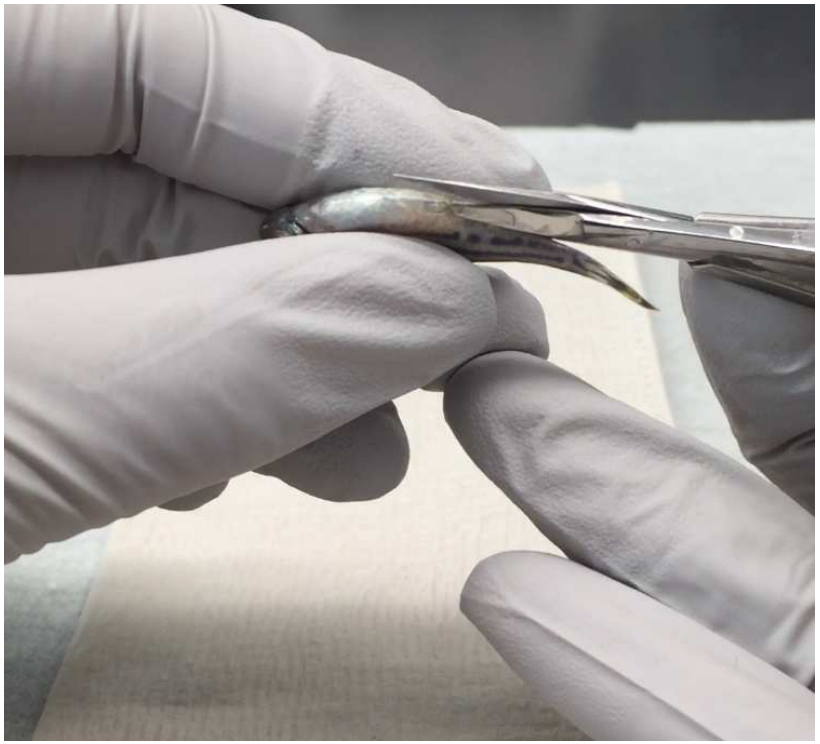
2. Remove fish from tricaine and blot excess water away.



Dissection and Fixation for Histology

3. Using micro scissors, cut open the coelomic cavity.

4. Using razor blade, cut tail off at the caudal peduncle region.



Dissection and Fixation for Histology

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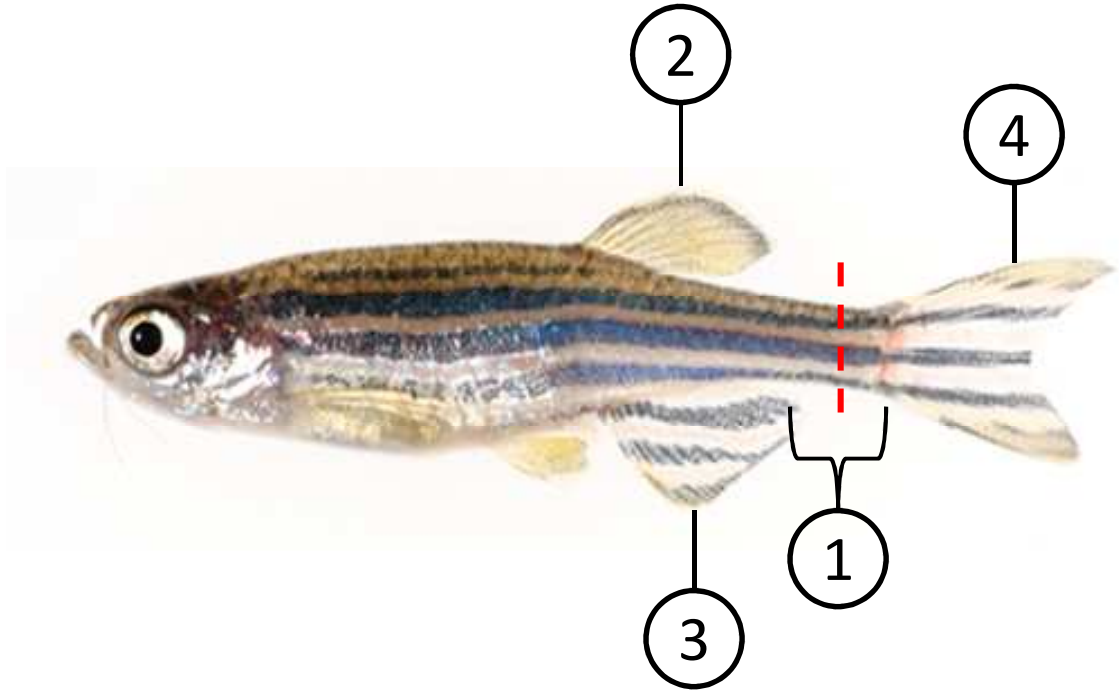
Dissection and Fixation for Histology



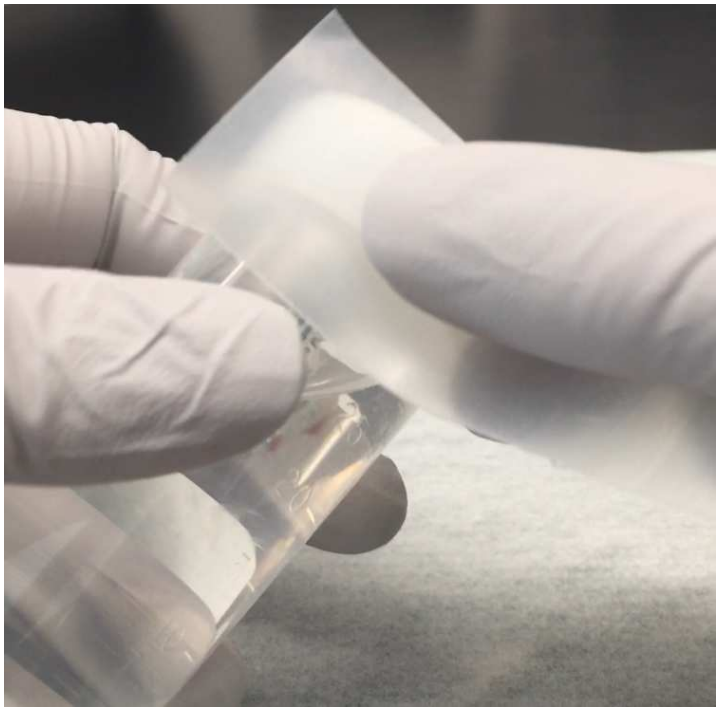
The 1) caudal peduncle is the tapered region behind the 2) dorsal and 3) anal fin where the 4) caudal fin attaches to the body.

Dissection and Fixation for Histology

5. Using forceps, drop fish into sealable container of fixative of choice.
   -volume is ~10-15mls of fixative per 1-2 fish

6. Strips of parafilm can be used to seal caps of tubes as a precautionary measure.

7. Place container of fixative and fish on rocker for 24 hours post fixation.



Surface Sanitation of Embryos

GOAL: To significantly reduce transmission of pathogens and parasites within a colony and during importation process of new lines.

Challenges: Not all pathogens and parasites are reduced or eliminated by all types of disinfection chemicals.

Solution: Optimize methods to maximize concentration, duration, and spectrum of organisms suppressed.

Embryo Bleaching - Supplies

Materials Needed:

- **5 small containers**
- **Embryo strainers**
- Clean fish water or EM
- RO water
- 5-6% scientific grade bleach
- Household bleach
- Sodium Thiosulfate

Embryo Bleaching - Supplies

Materials Needed:

- 5 small containers
- Embryo strainers
- **Clean fish water or EM**
- **RO water**
- 5-6% scientific grade bleach
- Household bleach
- Sodium Thiosulfate

RO Water

Embryo media
Or
Sterile fish water

Embryo Bleaching - Supplies

Materials Needed:

- 5 small containers
- Embryo strainers
- Clean fish water or EM
- RO water
- **5-6% scientific grade bleach**
- **Household bleach**
- **Sodium Thiosulfate**

Embryo Bleaching - Supplies

1. Sanitize all work surfaces and supplies with household bleach at a concentration of 500 ppm (500mg/liter)
   –neutralize with sodium thiosulfate and rinse well

2. Make all stock solutions (bleach water, rinse water, neutralizing bath)

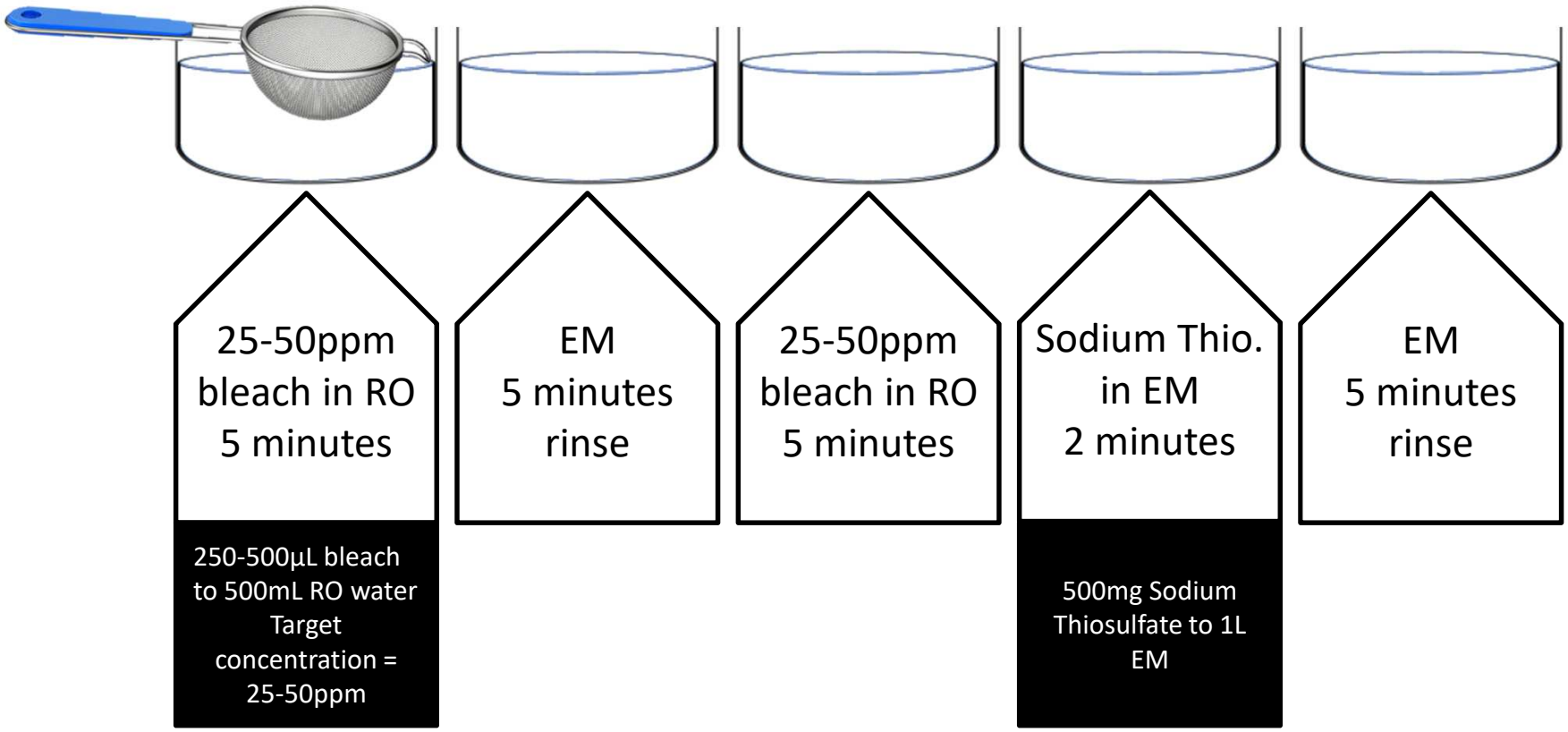
3. Set up bleaching station in the following configuration:

   Bleach 1 - Rinse 1 - Bleach 2 - Thio Rinse - Rinse 2

4. Pre-clean embryos, removing any that are dead or damaged

5. Rinse embryos into strainer

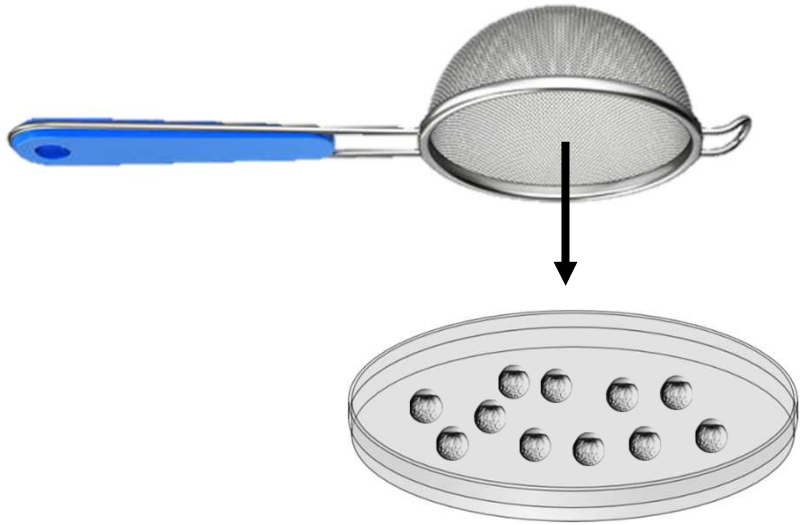
Embryo Bleaching - Protocol



Embryo Bleaching - Protocol

Rinse embryos into petri dish w/embryo media

- Keep in densities no greater than 50 per 100mm dish



Embryo Bleaching – Alt Methods and Considerations

4 Factors to Consider

Age of embryos

Concentration of bleach

Duration of exposure

Potential strain variability

- Some facilities utilize one 10 minute exposure bath vs two 5 minute baths
- Concentrations of up to 100ppm have been found acceptable for 6hpf embryos
- Higher concentrations and exposure durations are more lethal to embryos but can be more effective at reducing pathogen transfer
- Larger water volumes are recommended (allows particulate to fall away from embryo)
- Rinsing embryos of detritus and particulate is vital

Bleach + PVP-I

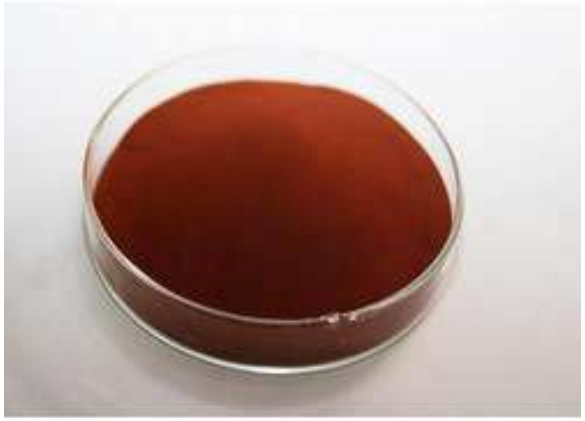
Materials Needed:

- **4 small containers**
- **Embryo strainers**
- Clean fish water or EM
- 6% scientific grade bleach
- **PVP-I (Providone Iodine)**

Bleach + PVP-I

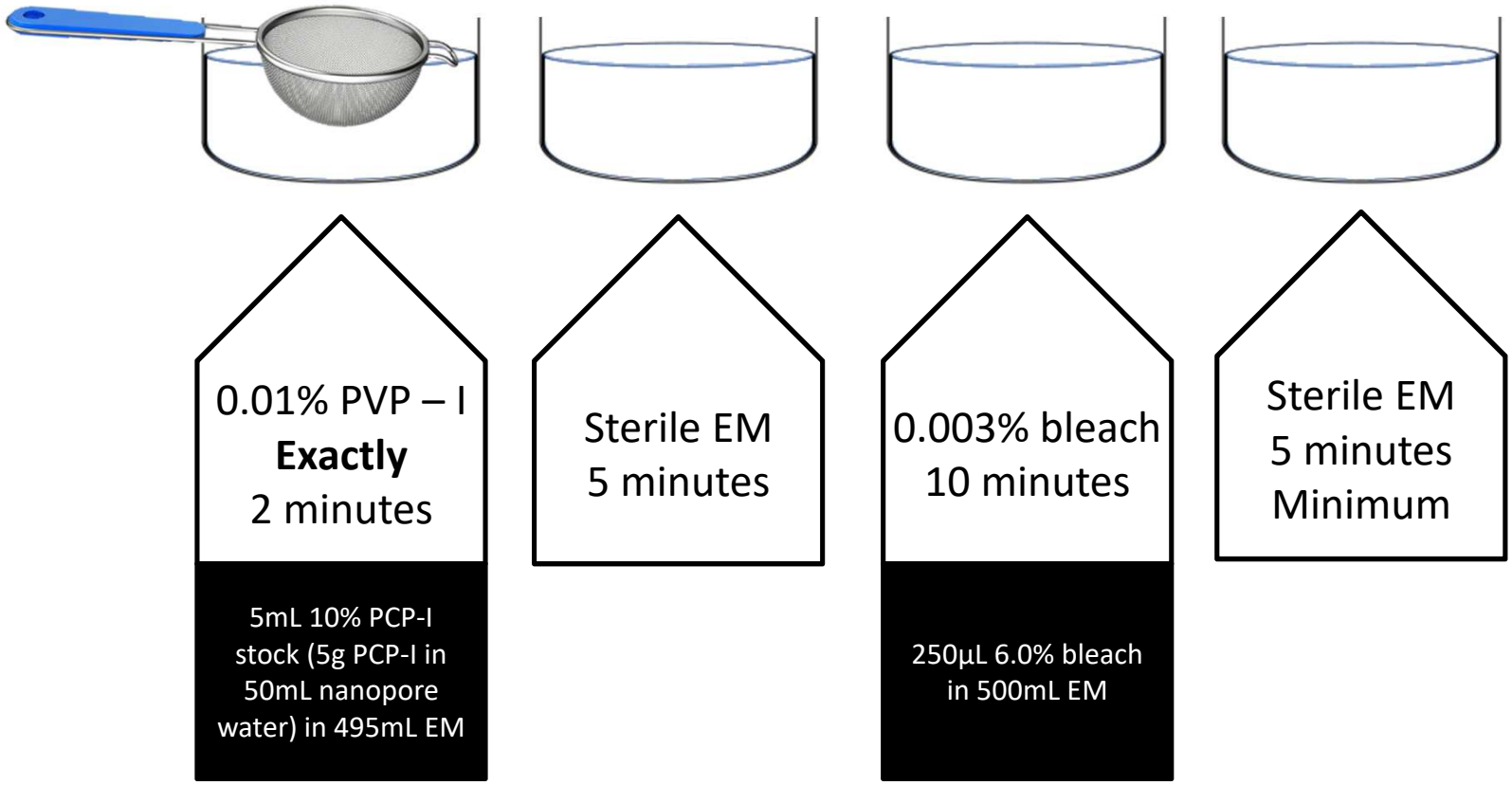
Materials Needed:

- 5 small containers
- Embryo strainers
- **Clean fish water or EM**
- **6% scientific grade bleach**
- **PVP-I (Providone Iodine)**



Embryo media

Bleach + PVP-I (Providone Iodine)



Shipping Fish - Supplies

Materials Needed:

- **ThermoSafe box**
- **Secondary containment bag**
- Cubitainer
- Absorbent bench towels
- Fish water
- Ammonia binder
- Heat packs
- Rubber bands or zip tie
- Live animal labels
- Appropriate documentation

Shipping Fish - Supplies

Materials Needed:

- ThermoSafe box
- Secondary containment bag
- **Cubitainer**
- **Absorbent bench towels**
- Fish water
- Ammonia binder
- Heat packs
- Rubber bands or zip tie
- Live animal labels
- Appropriate documentation

Shipping Fish - Supplies

Materials Needed:

- ThermoSafe box
- Secondary containment bag
- Cubitainer
- Absorbent bench towels
- **Fish water**
- **Ammonia binder**
- Heat packs
- Rubber bands or zip tie
- Live animal labels
- Appropriate documentation

Appropriate density for shipping is ~5 fish per liter

Shipping Fish - Supplies

Materials Needed:

- ThermoSafe box
- Secondary containment bag
- Cubitainer
- Absorbent bench towels
- Fish water
- Ammonia binder
- **Heat packs**
- **Rubber bands or zip tie**
- Live animal labels
- Appropriate documentation

Shipping Fish - Supplies

Materials Needed:

- ThermoSafe box
- Secondary containment bag
- Cubitainer
- Absorbent bench towels
- Fish water
- Ammonia binder
- Heat packs
- Rubber bands or zip tie
- **Live animal labels**
- **Appropriate documentation**

This almost entirely depends on the country of origin and destination

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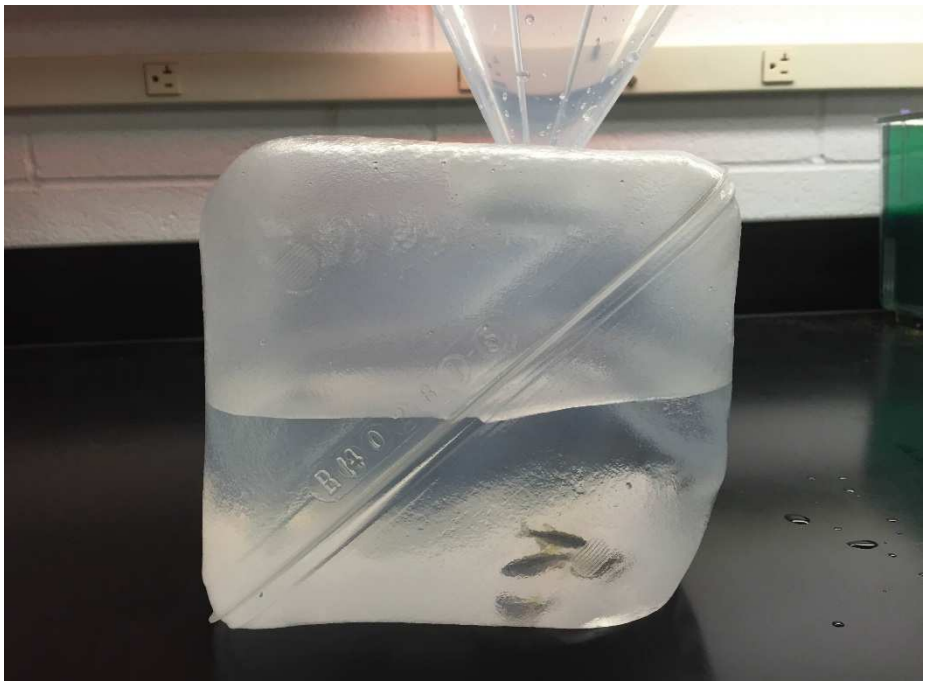
A SERVICE NO ONE ELSE CAN DELIVER

Shipping Fish - Procedure

1. Add ammonia binder to fish water
2. Fill the cubitainer no more than 1/3 full using funnel
3. Add fish and seal lid tightly

1 part water to 2 parts air space



Shipping Fish - Procedure

4. Place absorbent bench towel inside containment bag within the box

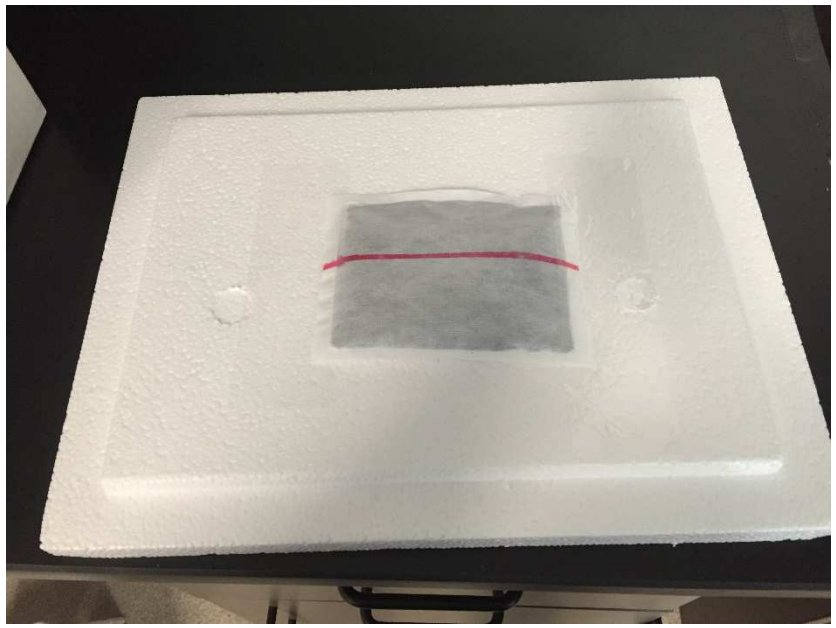
5. Nest cubitainer inside bag

Shipping Fish - Procedure

6. Twist the top of bag, knot, and fasten with zip tie or rubber band
7. Fill any additional space in box with non-absorbent packing material or bags/cubitainers of water

Shipping Fish - Procedure

8. Affix heat pack to lid, making sure it can't come in contact with water
9. Fit Styrofoam lid on to box, place copy of documents inside and seal



Shipping Fish - Considerations

Notes

- Separate fish to be shipped for several days prior to shipment for observation
- Withholding feed prior to shipment can help maintain water quality
- Some countries require a second or third set of documents to be fastened to the outside of the box in addition to the copy within the carton to be delivered to various agencies during transit
- Always label and declare that you are shipping live animals!

Shipping Fish - Considerations

The procedure for shipping embryos remains the same, but the containers and solutions are different.

In place of fish water and ammonia binder, use sterile fish water or embryo media w/methylene blue

In place of cubitainers, use 250 ml tissue culture flasks

- Fill flasks w/150-200ml EM
- Pipette up to 100 embryos per flask
- It is recommended that embryos be bleached

Receiving Fish - Warning

IMPORTANT NOTE!!!

During the shipping process, carbon dioxide released by the fish reacts with water to form a weak acid, which lowers pH. Therefor, even though ammonia levels can be high after transportation, it is a less toxic form.

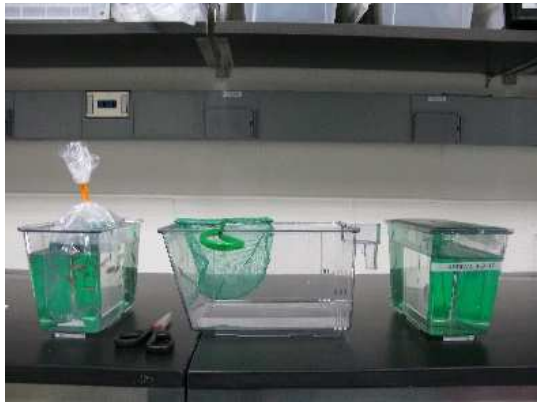
Opening the shipping bag causes the carbon dioxide to be released into the environment, resulting in a quick increase in pH and which causes a rapid increase in toxic ammonia levels.

DO NOT open the shipping bag until fish are ready to be transferred directly into fresh system water!

Receiving Fish - Supplies

Materials Needed:

- **Tank filled with system water to acclimatize fish**
- **Tank filled with system water to house the fish**
- **Empty tank**
- Net
- Scissors (if fish arrived in a bag)



Receiving Fish - Supplies

Materials Needed:

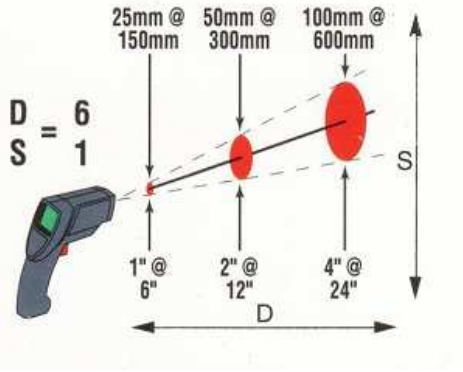
- Tank filled with system water to acclimatize fish
- Tank filled with system water to house the fish
- Empty tank
- **Net**
- **Scissors (if fish arrived in a bag)**

Receiving Fish - Supplies

An inferred temperature gun is an especially useful tool in a quarantine environment, if used correctly.

Inferred sensors can give an inaccurate reading if surface is reflective, therefor it is important to create a matte surface to measure at.

Know what the distance to spot diameter ratio of your IR Sensor is.



Receiving Fish - Procedure

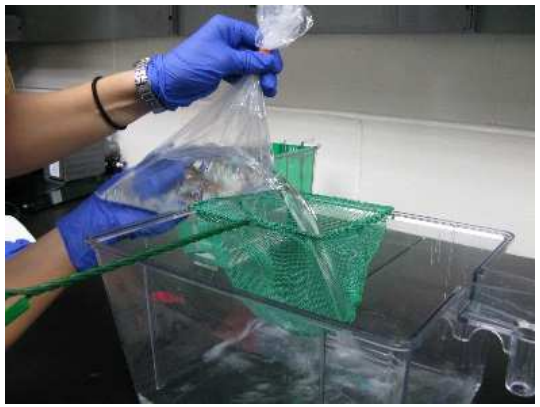
1. Acclimatize fish to the housing temperature off the system.

   -this is done floating the fish bag in a tank filled with warm system water

2. When bag is within 2 degrees of housing water, remove from acclimatization tank, and hold over net nested within empty tank.

Receiving Fish - Procedure

4. Cut corner of bag with scissors and pour fish into net.
5. Transfer fish from net into new housing tank w/water.
6. Label tank with line information and place on quarantine system.
7. Observe fish for signs of illness or distress
8. Discard shipping water and clean all supplies with approved quarantine protocol



Thank You

Oregon State University

EHSQ new discoveries

TANGUAY LAB
THINKING OUTSIDE THE TANK