Zebrafish Procedures

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topics to cover

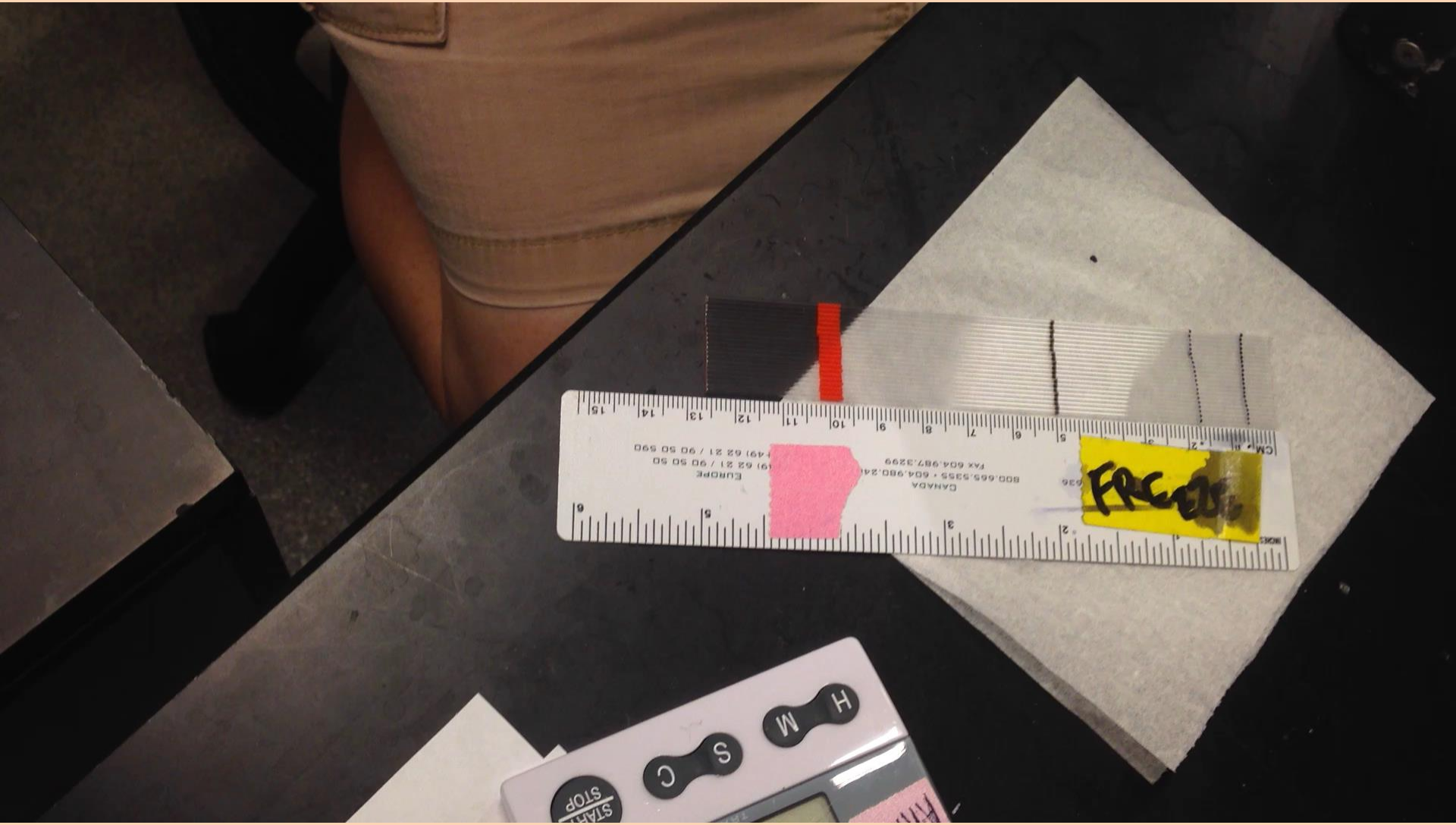
- Cryopreservation
- IVF
- Dechorionation
- Bleaching of embryos
- Prophylactic Drug Treatment of incoming fish
- Fin Clipping
- Genetic Screens
- ENU Mutagenesis
- Micro-injection
- Artemia Decapsulation
- Shipping Fish
- Tank Washing

Common Procedures in Zebrafish Facilities

– sperm collection / cryopreservation

http://www.jove.com/video/1395


10µL
Cat. No. 2-000-010
Made in USA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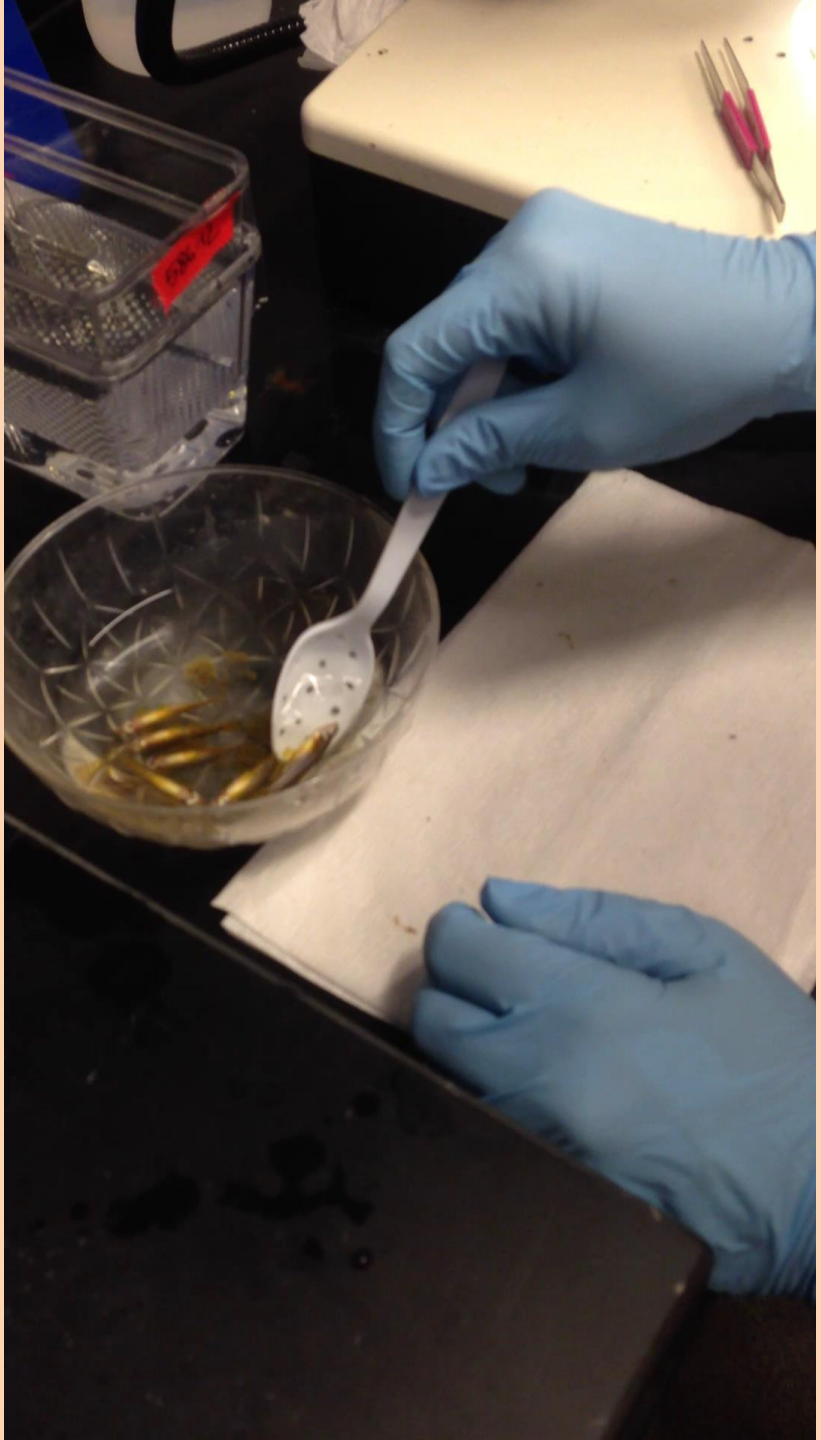
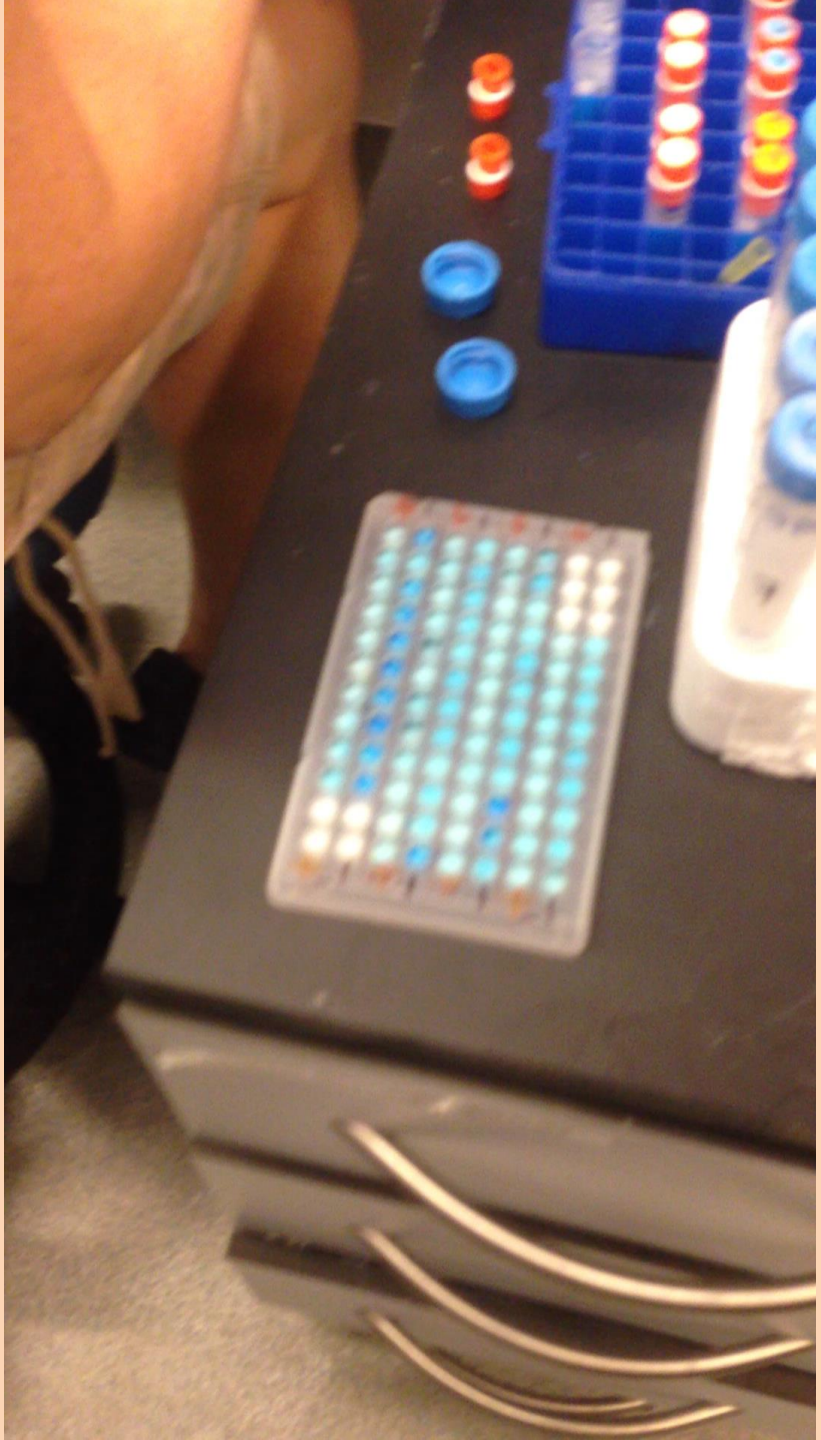




Common Procedures in Zebrafish Facilities

- In Vitro Fertilization
  - maintenance squeeze
  - sickly/picky lines
  - cryo-preservations
  - synchronized embryos

Why IVF?

- If the females are gravid, the eggs can be had without waiting for the female to choose to spawn.
- With practice, fertilization averages +90%
- Fish experience less egg-binding
- Makes ID of un-cooperative fish possible.
- Arguably a faster means of getting embryos.

In Vitro Fertilization – set up of fish

- **Over/Under Breeder Tanks**
- **♀3:♂2**
- **At least 2 large pieces of plant**
- **Water directly from system?**
- **Performed at end of day ~PM**

Fish Selection for IVF

- **Females selected first if “sweet spot” (anterior of vent was slightly red and swollen) was present.**
- **Males chosen for overall color and healthy appearance**

IVF Procedure

The Station

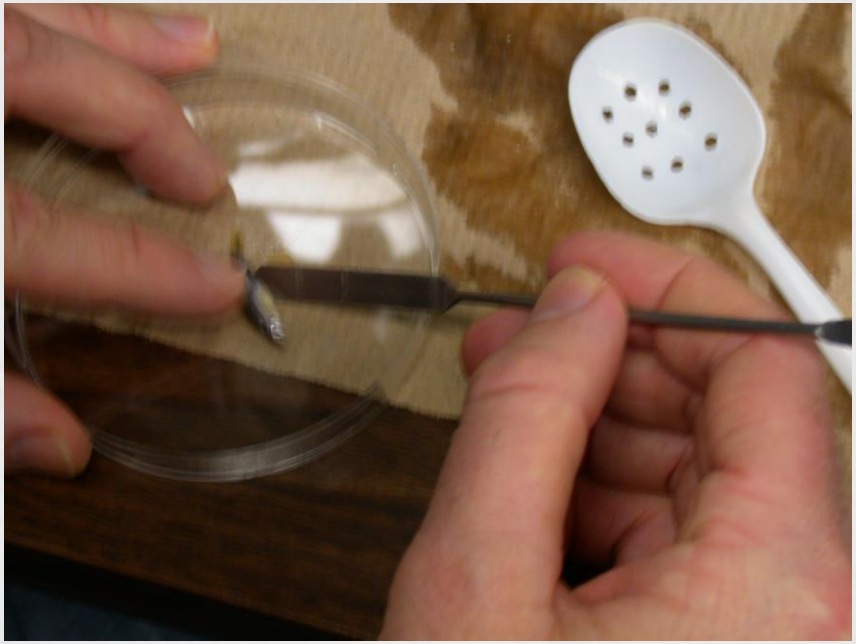
- Stereo Microscope w/ sponge
- Bowls of system water and bottles of concentrated tricaine (20X)
- Rescue bowls
- Paper towels
- Drilled spoons
- Capillary tubes (Drummond 20uL)
- Lab spatula
- Petrie dishes for eggs
- Labeled containers for fish held pending ID

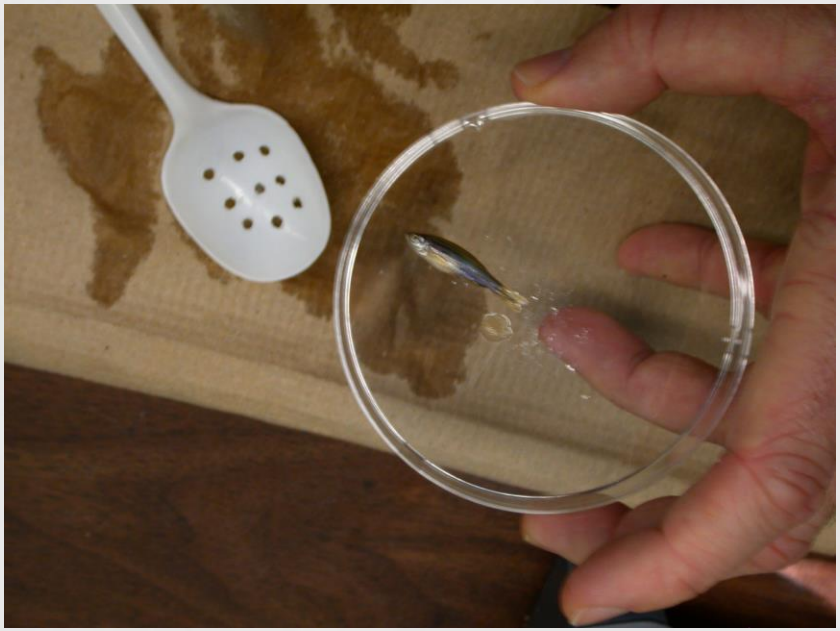
IVF Procedure- females

- **Females are anesthetized to loss of righting response.**
- **Removed from tricaine with drilled spoon**
- **Flipped and rolled on paper towel to dry**
- **Scooped and placed in Petri dish**
- **Gentle pressure in front of vent**

IVF Procedure- females (continued)

- Eggs separated from fish with spatula
- Female flicked to rescue tank



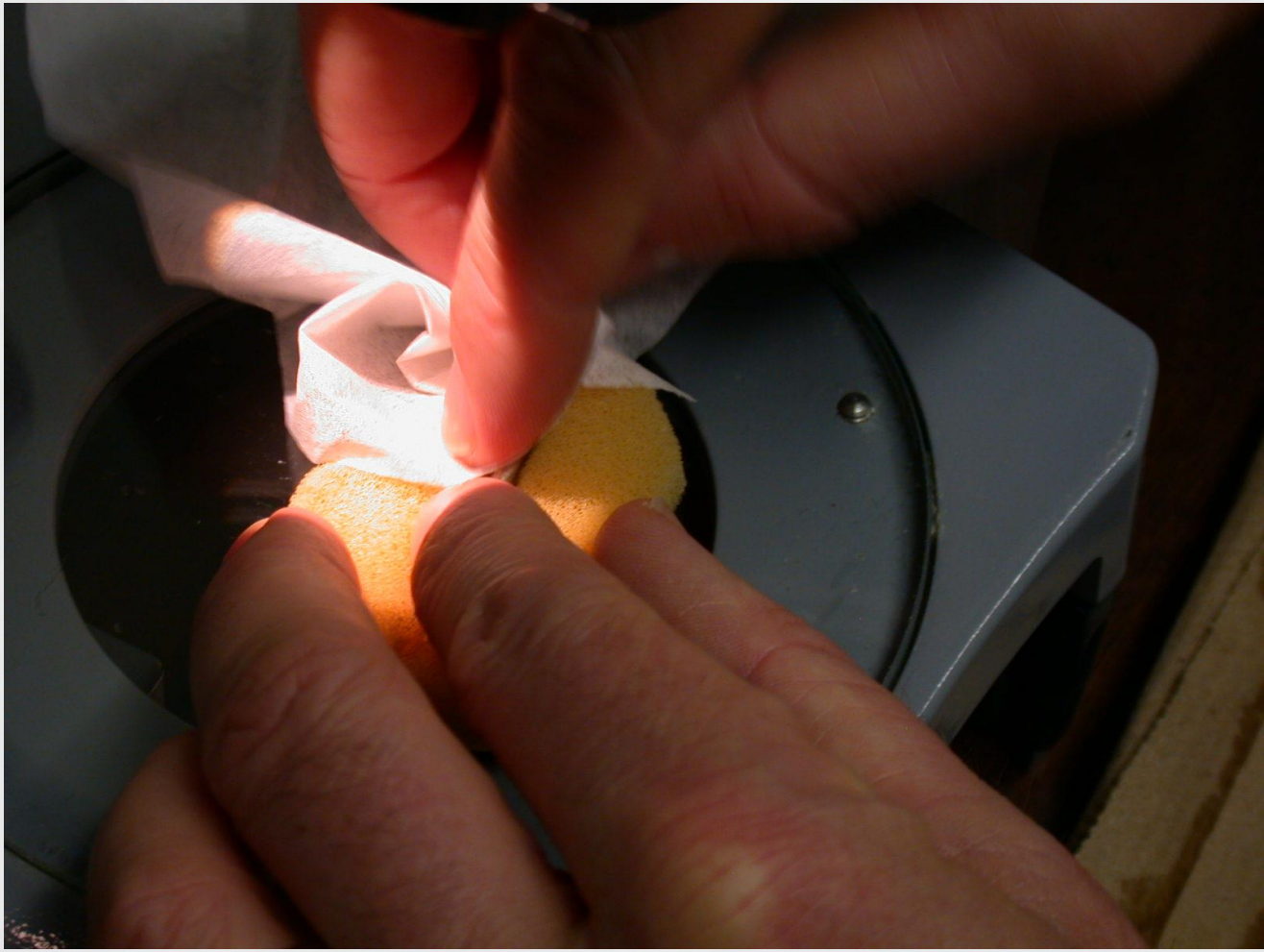


Collected Eggs

- Eggs of females are collected and stored in capped Petri dishes.
- It is not uncommon to have 15-30 dishes awaiting fertilization.
- It has been reported that these eggs are still viable up to an hour after squeezing.

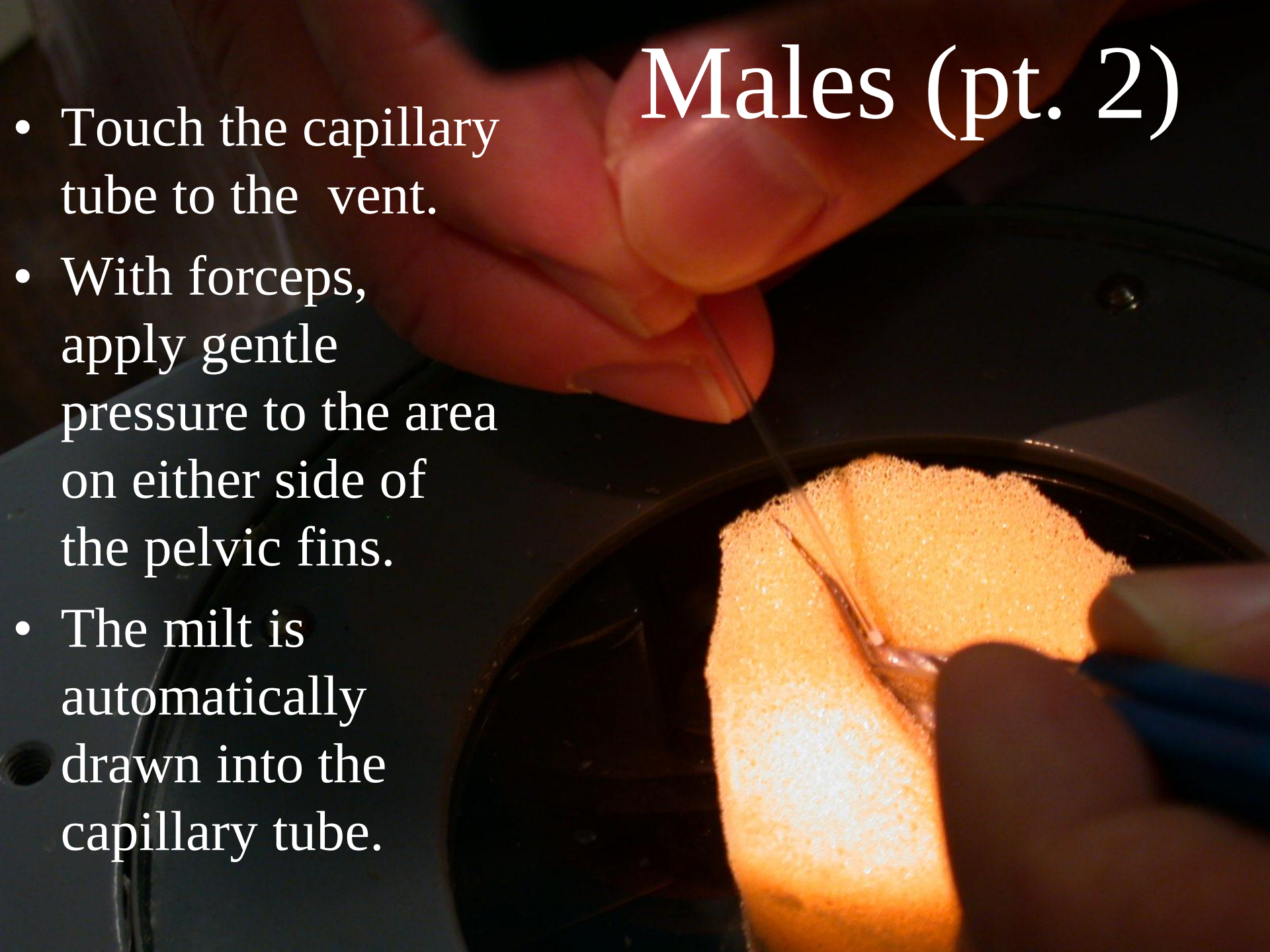
Male IVF Procedures

- Males are anesthetized in the same manner as females.
- Placed on dorsal side in sponge with notch.
- Area around pelvic fins/vent dabbed dry with Kimwipe.



Males (pt. 2)

- Touch the capillary tube to the vent.
- With forceps, apply gentle pressure to the area on either side of the pelvic fins.
- The milt is automatically drawn into the capillary tube.



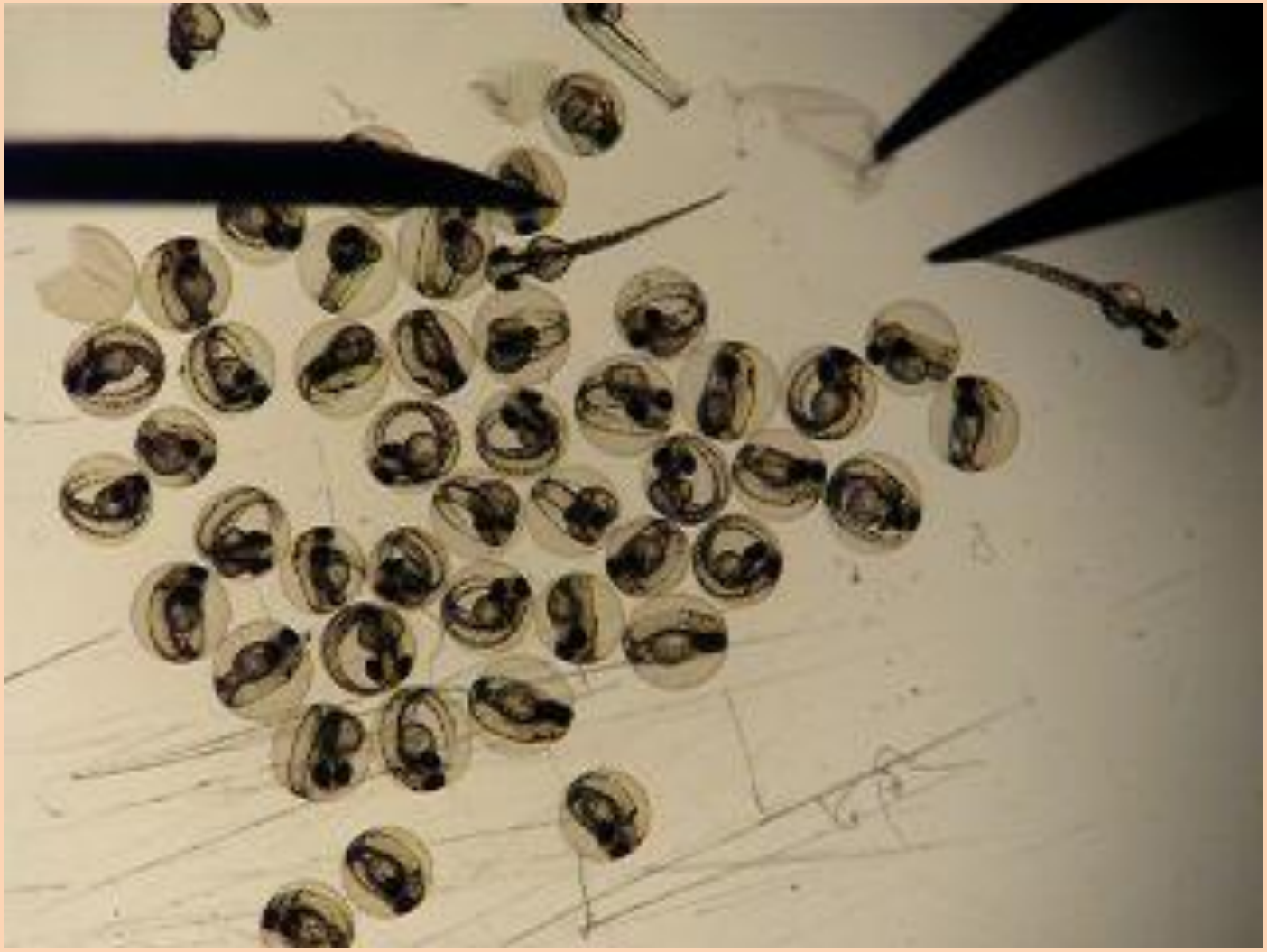
Milt

- Typically used at once by blowing the capillary tube clear. Then a few drops of system water added to “activate”.
- Can be frozen and archived.
- One male often provides enough milt to fertilize 2-5 clutches of eggs.

Males Held Pending ID

- 250mL beakers
- 75-100mL water
- Numbered
- Second tray serves as lid.

Manual Dechorionation of Embryos



Chemical Assisted Dechorionation of Fertilized Embryos

Pronase Dechorionation:

The bleach treatment makes the chorion harder and the embryos will not be able to hatch on their own. Therefore they must be manually dechorionated with forceps, or with pronase .

Solutions Required:
Pronase 20mg/ml (Sigma P-5147)

add 1mL of Pronase to a Petri dish of up to 250 embryos (in egg water) for exactly 3 minutes
pour off Pronase containing egg water and refill with autoclaved egg water.
Repeat this step for a total of 3 washouts.
Refill dish with autoclaved egg water.

Some embryos will have hatched immediately, others may require 24-hrs to hatch, or can be hatched by gently swirling or pipetting up and down with a transfer pipette.

Bleach Disinfection of Fertilized Embryos (~24-hpf)

Embryo Bleaching:

Every new line to be introduced to the fish facility must be treated with bleach solution in order to reduce transfer of potential pathogens. Only bleached embryos can go into the system of the main fish facility.

Bleaching is best done between 24-30 hpf.

Solutions Required:
Bleach solution= 700 μL bleach/L autoclaved egg water.
Autoclaved Egg Water (

submerge embryos in bleach solution for exactly 5 minutes
remove embryos from bleach solution and submerge in autoclaved egg water for 5 minutes
Repeat step 1
Repeat step 2
5 minutes in egg water
5 minutes in egg water

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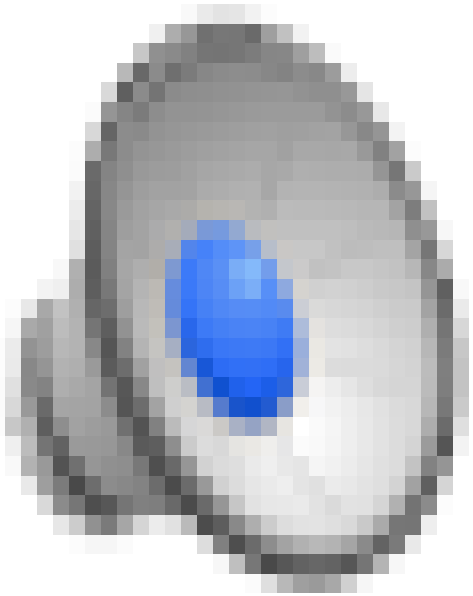
Fin Clip Genotyping



THE ART AND DESIGN OF GENETIC SCREENS: ZEBRAFISH

Elizabeth Patton and Leonard I. Zon

Inventive genetic screens in zebrafish are revealing new genetic pathways that control vertebrate development, disease and behaviour. By exploiting the versatility of zebrafish, biological processes that had been previously obscured can be visualized and many of the responsible genes can be isolated. Coupled with gene knockdown and overexpression technologies, and small-molecule-induced phenotypes, genetic screens in zebrafish provide a powerful system by which to dissect vertebrate gene function and gene networks.

ENU Mutagenesis

Please keep in mind that ENU is a potent alkylating agent, and thus a mutagen. Exert extreme caution when dealing with it, keeping in mind both your own safety and of those around you. Please wear protective clothing (lab coat, non-sandal shoes, booties on your shoes, face mask, doubled gloves) and make sure that anything with traces of ENU on it is decontaminated by incubation is sodium thiosulfate bath.

Mutagenesis:

Egg water (2.4 g Instant Ocean in 8L of ddH_20) buffered using a solution of 100 mM sodium phosphate added to the egg water to achieve pH 6.5 (pH meter). Cool down to 20-21°C on ice to prepare for mutagenesis – (1L- volume of ENU solution). Add 5.0 mL of 4 g/l MS-222 (tricaine/MESAB) stock per 1L of mutagenesis buffer for anesthetization.

Prepare hood for mutagenesis: cover surface with black material (large trash bag), transfer mutagenesis tanks (20-21°C) to hood, add ENU stock (Z ml) to 1l of mutagenesis buffer and switch off all lights and noise sources (e.g. water circulation of fish system). Transfer fish from a system tank to a transfer cage and very carefully/quietly transfer the insert with fish to mutagenesis tank; cover with plastic plate. Note time immediately and mutagenize for 1 hour. Leave the room and prepare recovery buffer in the mean time (17-19°C, 10 mg/l MESAB).

Recovery

Cool down the prepared egg water below 19°C (17-19 °C) on ice for recovery. Add 5 mL of 4 g/l MS-222 (tricaine/MESAB) stock per 1L of recovery water for anesthetization.

Prepare ENU decontamination tank: Dissolve 1,250 g Sodium Thiosulfate in 12.5L of tap water, buffer with 5 M NaOH to pH ~10 (pH-strips).

ENU Mutagenesis

Suggested Mutagenesis/Recovery Schedule:

Day 1

9AM	preparations
10AM -12PM	dissolving ENU
12.00PM -1PM	treatment of males
1PM - 7PM	recovery in MESAB (Transfer #1)
7PM	transfer to a recovery solution without MESAB (Transfer #2)

Day 2

Transfer #3 into egg water

Transfer #4 into system water from facility and transfer to facility

Common Procedures in Zebrafish Facilities

- major components of the microinjection system:
  - The stereoscope
  - microinjector
  - pipette holder
  - micromanipulator

URL: http://www.jove.com/science-education/5130

Common Procedures in Zebrafish Facilities

- MicroInjection
  - drugs, markers, RNA, DNA, etc.
  - prep work is +99% of success
  - practice, practice, practice!
  - synchronized embryos

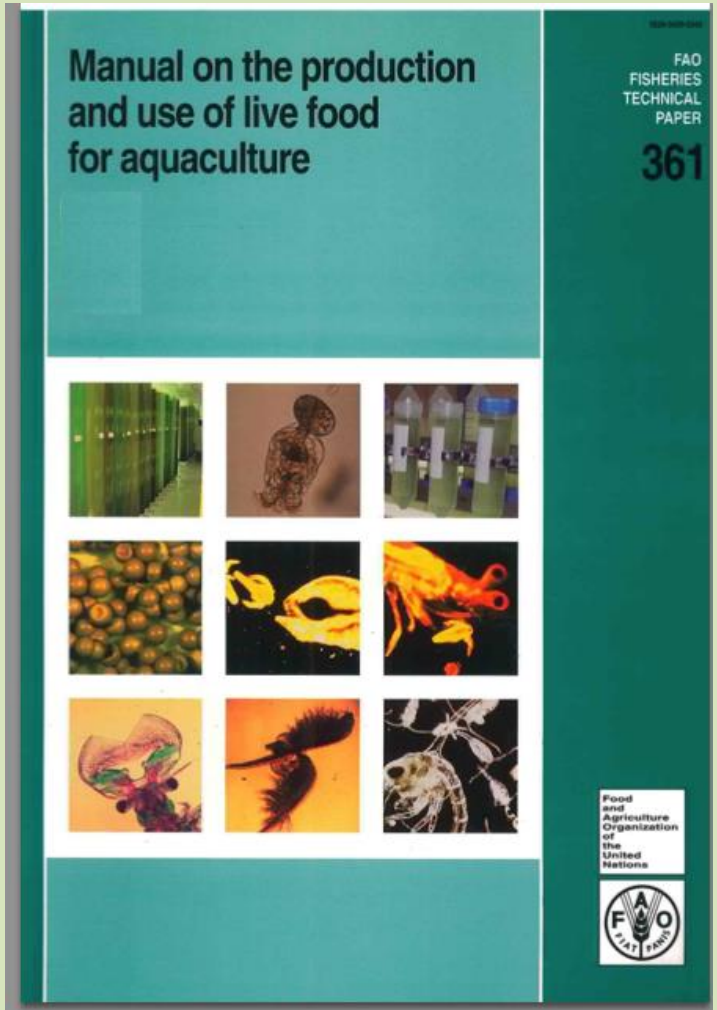
Common Procedures in Zebrafish Facilities

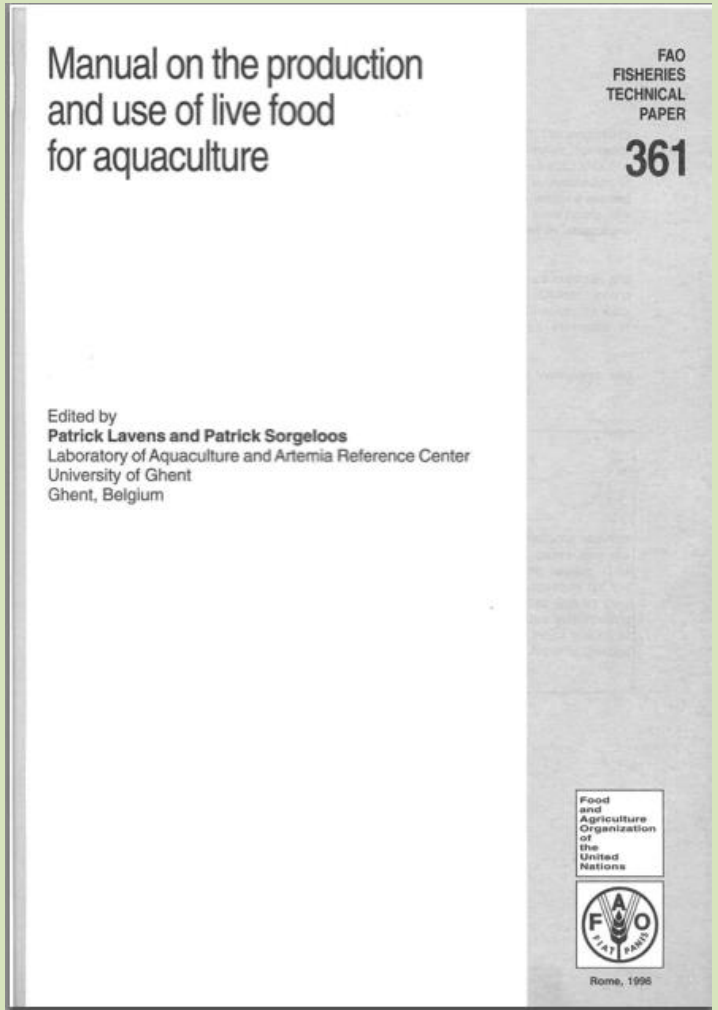
- Decapsulation of Artemia Cysts
  - removes tough, protein shell
  - disinfects
  - aids in hatching
  - streamlines daily work
  - large batches possible

Common Procedures in Zebrafish Facilities

Decapsulation of Artemia Cysts

- get the FAO document (1996) and read the artemia section (170-pages!!)





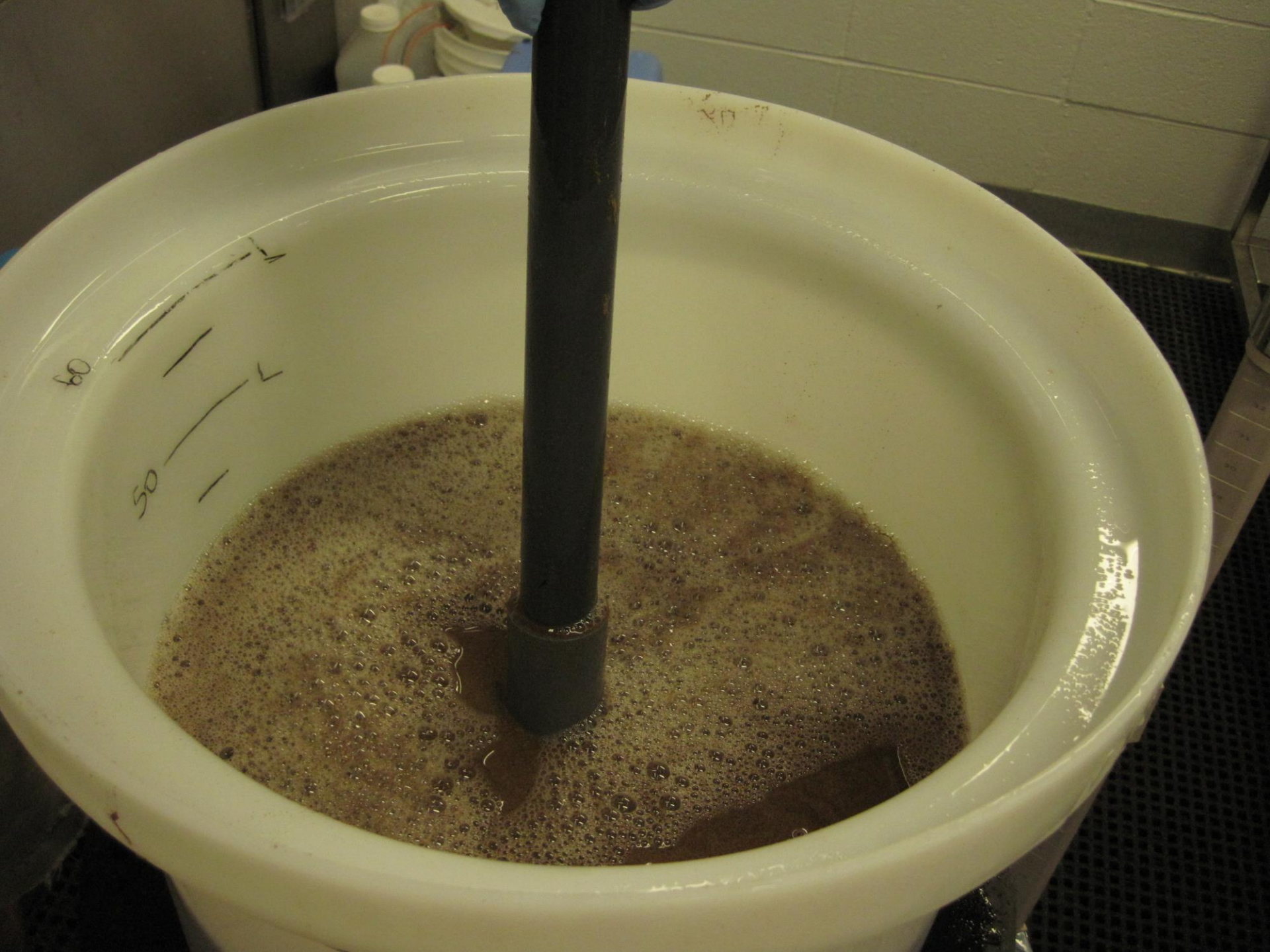
Common Procedures in Zebrafish Facilities

- Decapsulation of Artemia Cysts
  - hydrate with tap water (~90-min)


GSL-INT
Sweepest for Nature and Life
PREMIUM ARTEMIA CYSTS


60
50 L
40 L
10

80
50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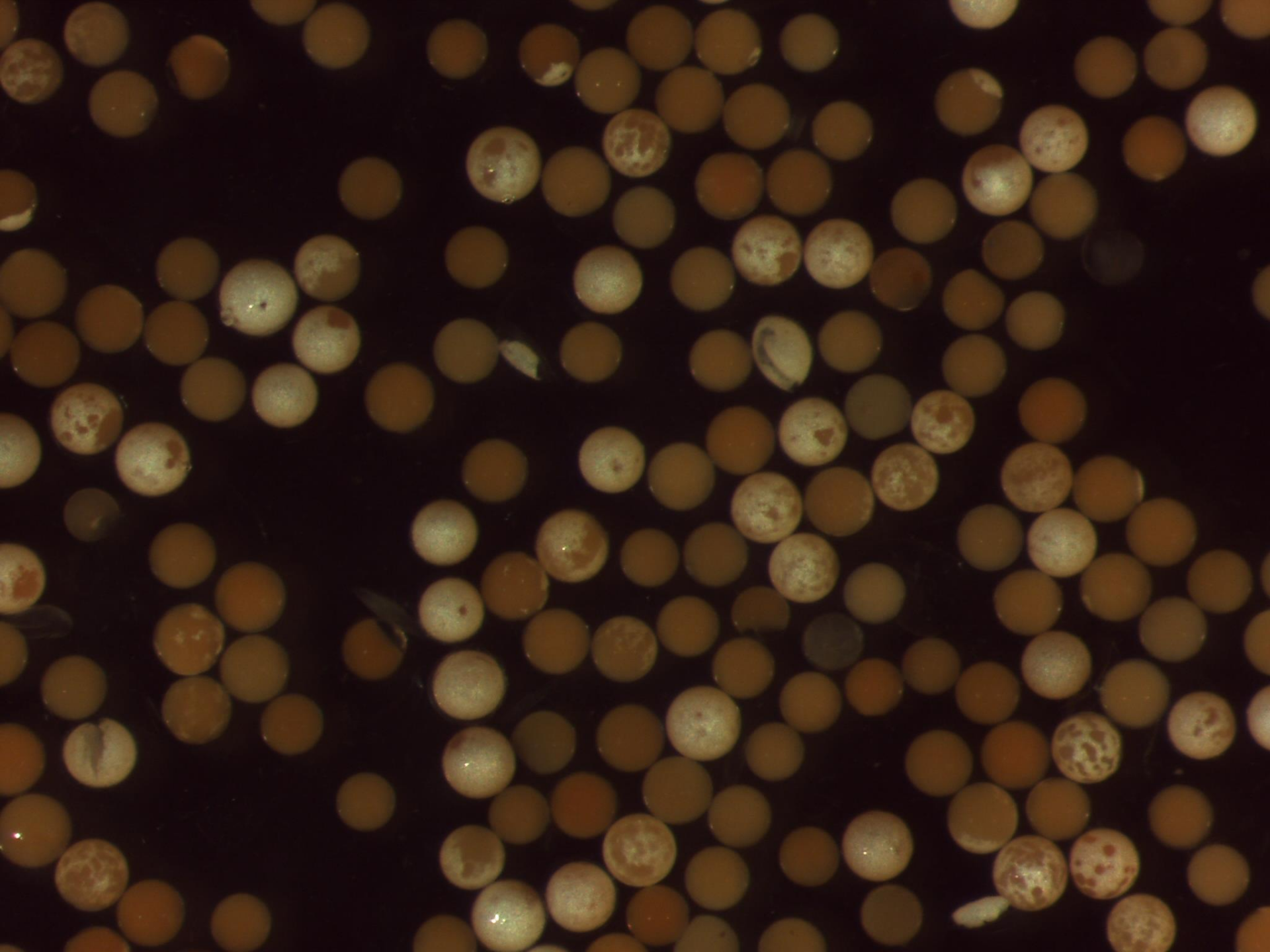



Decap. Brine shrimp
200ml/cone

	artemia cysts (g)	hatcher size (L)	hatch concentration (g/L)	feedout concentration (g/L)	qty tanks fed	approx. qty fish	approx. (g) artemia/fish	% of ave body mass (0.5g)
VAND	36	20	1.80		1230	14400	0.00250	0.50%
VAND	34	14	2.43		1230	14400	0.00236	0.47%
WASHU old	206	103	2.00	9	4800	144000	0.00143	0.29%
WASHU new (may22)	412	104	3.96	9	4800	144000	0.00286	0.57%
BOSTON	350	35	10.00		2000	125000	0.00280	0.56%

1-L bottles needed									
18	908	1029	1087	1178	1275	1456	1543	1641	1734
18.5	933	1057	1117	1210	1311	1496	1586	1687	1782
19	958	1086	1147	1243	1346	1536	1629	1733	1830
19.5	983	1114	1177	1276	1381	1577	1671	1778	1878
20	1008	1143	1207	1309	1417	1617	1714	1824	1926
20.5	1034	1171	1237	1341	1452	1658	1757	1869	1974
21	1059	1200	1268	1374	1488	1698	1800	1915	2022
21.5	1084	1229	1298	1407	1523	1739	1843	1960	2071
22	1109	1257	1328	1439	1558	1779	1886	2006	2119
22.5	1134	1286	1358	1472	1594	1819	1929	2052	2167
23	1160	1314	1388	1505	1629	1860	1971	2097	2215
23.5	1185	1343	1419	1538	1665	1900	2014	2143	2263
24	1210	1371	1449	1570	1700	1941	2057	2188	2311
24.5	1235	1400	1479	1603	1736	1981	2100	2234	2360
25	1261	1429	1509	1636	1771	2022	2143	2280	2408
25.5	1286	1457	1539	1668	1806	2062	2186	2325	2456
26	1311	1486	1569	1701	1842	2102	2229	2371	2504
26.5	1336	1514	1600	1734	1877	2143	2271	2416	2552
27	1361	1543	1630	1767	1913	2183	2314	2462	2600
27.5	1387	1571	1660	1799	1948	2224	2357	2508	2648
28	1412	1600	1690	1832	1983	2264	2400	2553	2697
28.5	1437	1629	1720	1865	2019	2305	2443	2599	2745
29	1462	1657	1751	1897	2054	2345	2486	2644	2793
29.5	1487	1686	1781	1930	2090	2385	2529	2690	2841
30	1513	1714	1811	1963	2125	2426	2571	2736	2889
30.5	1538	1743	1841	1996	2161	2466	2614	2781	2937
31	1563	1771	1871	2028	2196	2507	2657	2827	2986
31.5	1588	1800	1901	2061	2231	2547	2700	2872	3034
32	1613	1829	1932	2094	2267	2588	2743	2918	3082
decap density (g/mL)	0.170	0.150	0.142	0.131	0.121	0.106	0.100	0.094	0.089

Shipping Fish

On October 29, 2010, two packages, each containing a bomb consisting of 300 to 400 grams (11–14 oz) of plastic explosives and a detonating mechanism, were found on separate cargo planes.

They were bound from Yemen to the United States, and were discovered at enroute stop-overs, one **at East Midlands Airport in the UK** and one in Dubai in the United Arab Emirates.

Fish were shipped:

- double-bagged
- 4-bags of fish
- 6-10-fish/bag
- ~3L of filtered system water (20um)
- ~2/3 water 1/3 room air
- food with-held for 5-7 days
- free space in the styrofoam box was filled in with bags of water (Thermal-Mass!)

- These fish took 2-days to get to UK (though overnight was selected)
- Then spent 7-days in the Customs Office
- Then took 2 more days to get to Edinburgh
- 11-days in transit
- only 2-fish died

Fish were shipped:

- ~~•double-bagged~~
- ~~•4-bags of fish~~
- •6-10-fish/bag (
- ~~•~3L of filtered system water (20um)~~
- •~2/3 water 1/3 room air
- •food with-held for 5-7 days
- •free space in the styrofoam box was filled in with bags of water (Thermal-Mass!)

tank wash methods currently employed in fish facilities

- 100% Manual
- manual with mechanized assist (e.g. Barmaid washer)
- manual with chemical (e.g. bleach, virkon, iodophore, etc.)
- manual followed by sanitizing rinse (~185F) in mechanical washer
- washing with chemical detergent (rare, poorly characterized)

Drawbacks to manual washing

part or component washed	average cost per unit
3.5L tanks	$0.369
all size bffles	$0.040
8L tanks	$0.469
all size lids	$0.076
siphon and spring	$0.071
1.1L tanks	$0.283
average	**$0.218**

Labor costs

drawbacks to manual washing

- Footprint
- soaking of tanks
- large volumes of water
- specialized wash stations and sinks required

drawbacks to manual washing

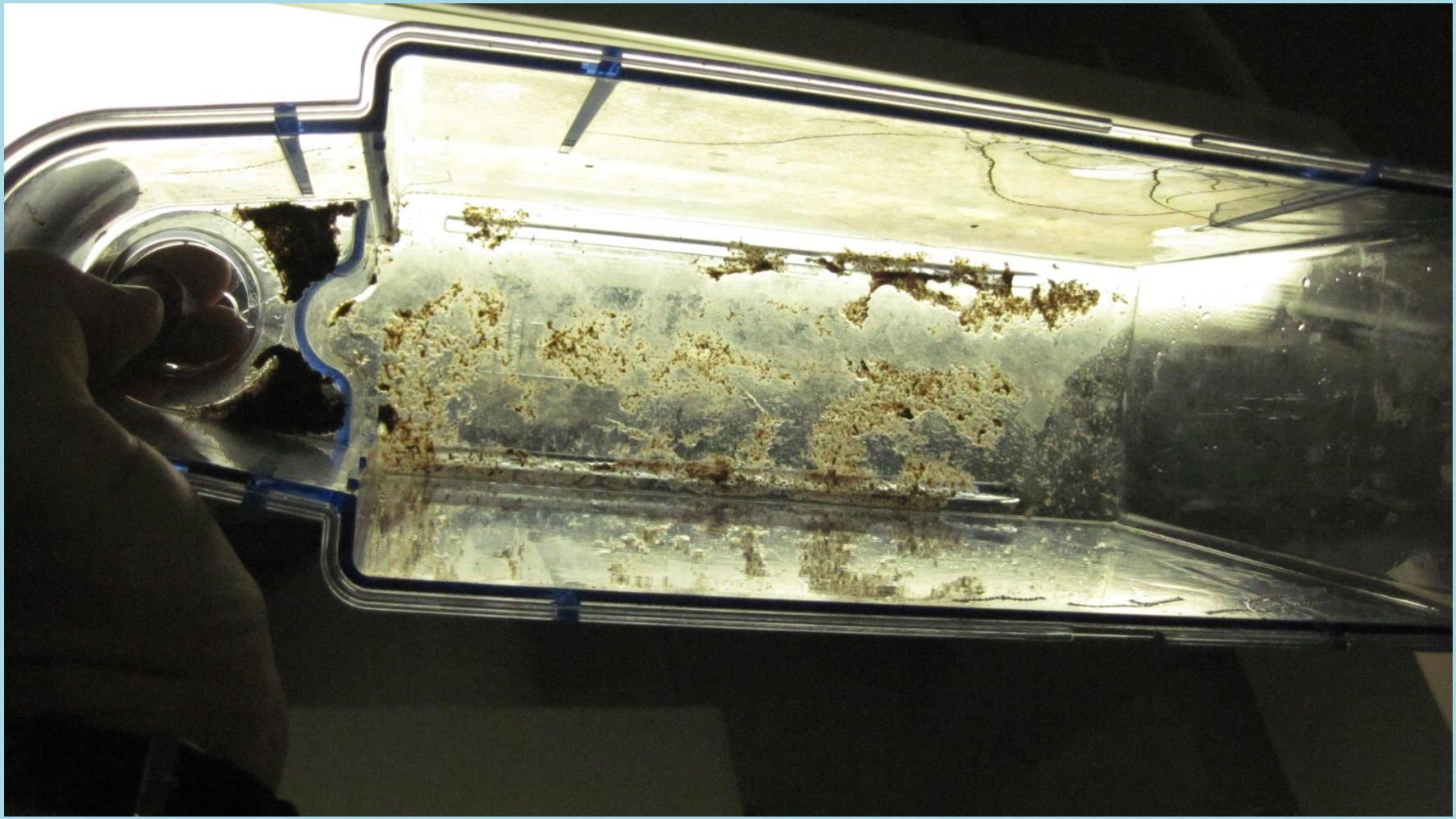
damage to tanks and components

- abrasion of surfaces by brushes or (non)abrasive pads, results in increased surface area in which biofilms and algae/diatoms can find purchase
- decreased service life of tanks and components

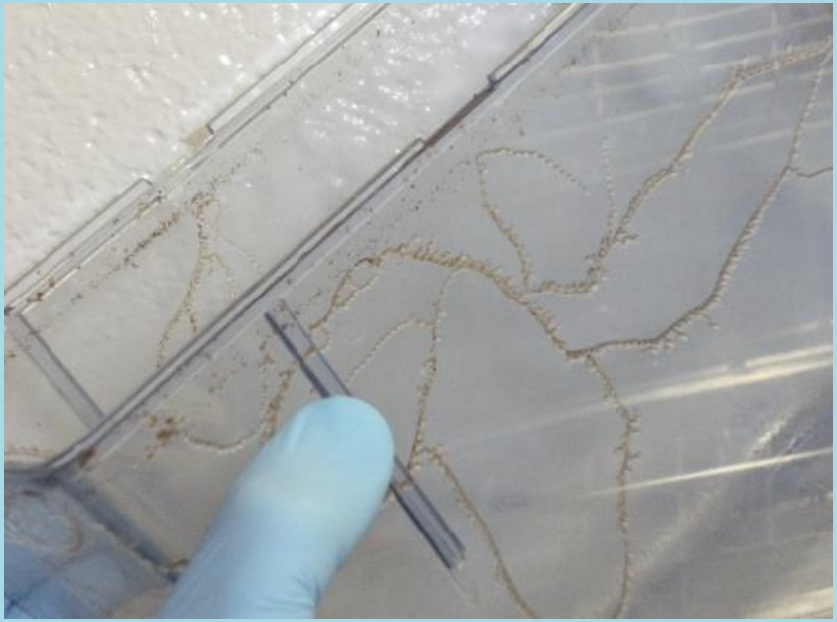
drawbacks to manual washing

- Use of chemicals commonly used in manual washing carry inherent risks
  - personnell risks
    - immunological sensitivity
    - skin sensitivity
    - respiratory sensitivity
      - (chlorine bleach)
  - destruction of shoes and apparel
  - cleaning chemical and/or neutralizer can be toxic to fish
  - as little as 2ppb is stressful to fish
  - disposing of large amounts of bleach, thiosulfate, or other chemicals may fall under the jurisdiction of local or federal guidelines

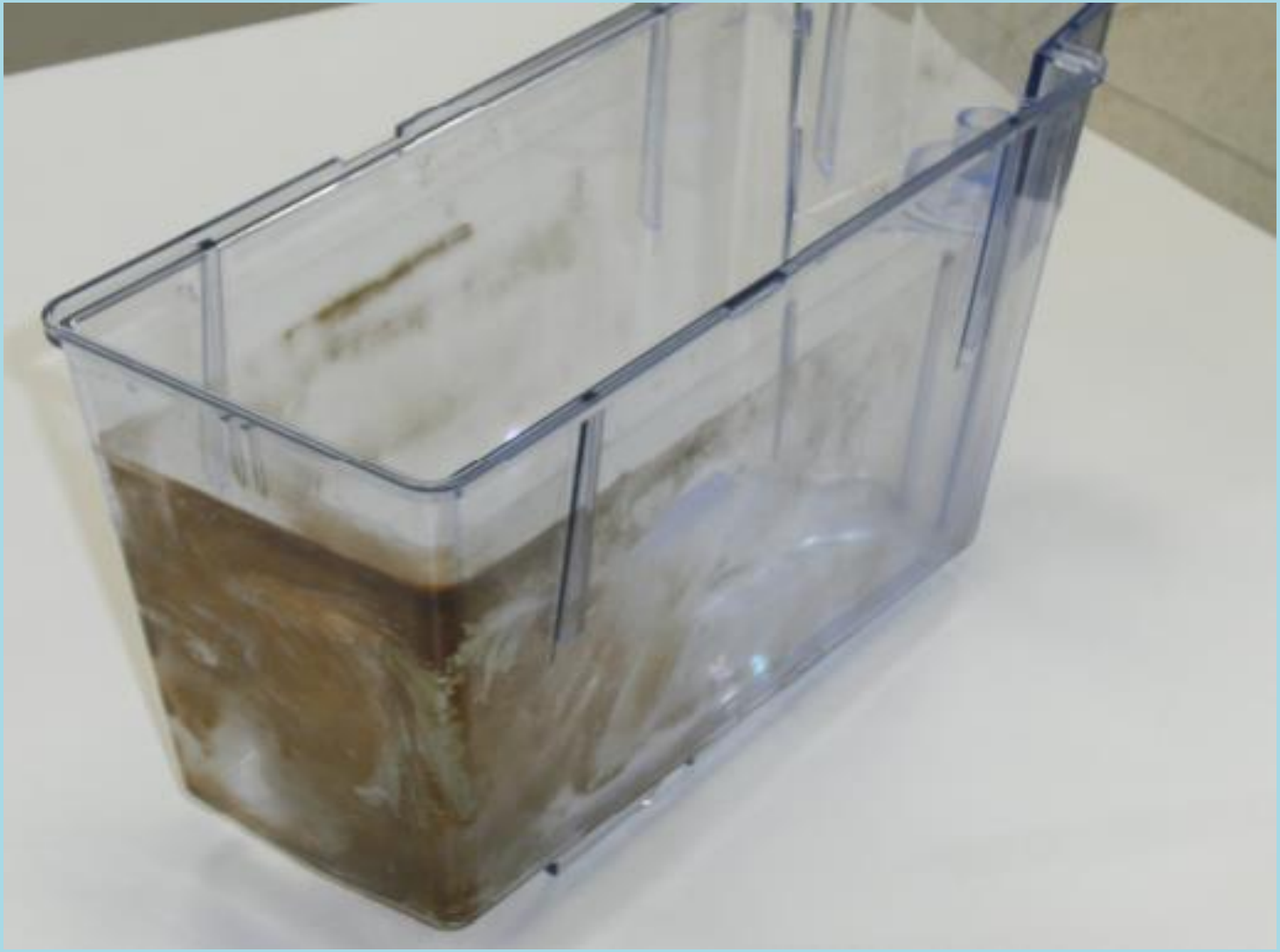
Why is Automated Washing so difficult? - biofilm (plaque)



Why is it so difficult? - bryozoan



Why is it so difficult? – algae/diatom



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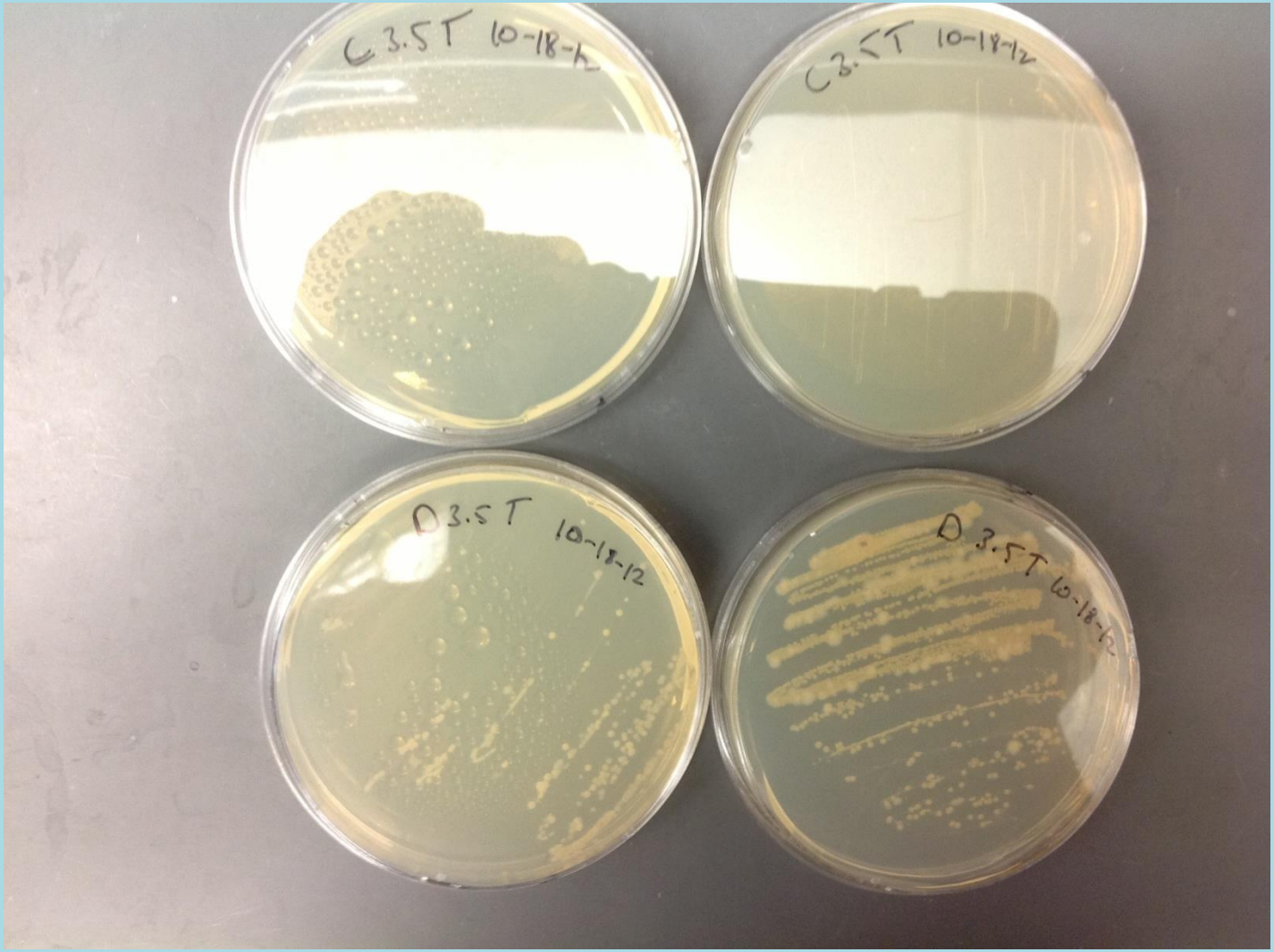
Validation of wash cycle- LB



3.5L tanks - clean

3.5L tanks - dirty

Productivity Comparisons - Manual vs. Automated Wash

part or component washed	average cost per unit	cost per unit in 650A	cost difference (%)
3.5L tanks	$0.369	$0.111	-70.0%
all size bffles	$0.040	$0.006	-84.8%
8L tanks	$0.469	$0.222	-52.7%
all size lids	$0.076	$0.055	-27.6%
siphon and spring	$0.071	$0.006	-91.5%
1.1L tanks	$0.283	$0.043	-84.8%
average	**$0.218**	**$0.074**	-66.2%

Thanks & Acknowledgements

Dr. Lila Solnica-Krezel, Stephen Canter, Chris Lawrence, Carole Wilson, Jason Cockington, Michael Kent

Zebrafish Consortium
Washington University in St. Louis

TECNIPLAST
Innovation through passion℠

Marco Brocca, Dillon Streets