

Follicle Handbook

Woodruff and Shea Labs

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McCormick

Northwestern Engineering



NORTHWESTERN
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****Please Note:** All protocols listed are what we currently use for mice: human/baboon/monkey protocols are different. Many protocols are also specific to the equipment used in the Woodruff and Shea labs – your lab may differ slightly**

RECIPES

Alginate (Sterile Aliquoting)

(Revised: June 25, 2007, MX)

To avoid sterile filtering alginate by using syringes, we developed a way to sterilize large quantities of alginate at once for later reconstitution.

1. Dissolve alginate powder in MilliQ H₂O to 0.5%-1% overnight with continuous stirring. Add 0.5 g activated charcoal per gram of alginate, stir for 30 min and then let settle (or spin in 50 mL tubes to accelerate). Sterile filter through Millipore 0.22 µm Express filters.
2. Transfer sterile alginate solution to 50ml steriflip tube (unscrew the filter membrane, transfer ~45 mL alginate solution into tube, and then re-screw the filter membrane, which will keep the alginate sterile for the following steps). Freeze down to -20°C (takes 1-2 days).
3. Lyophilize to dryness- takes ~5 days. Place the filter in a lyophilization jar in case the sterile filter pops (which can happen if the vacuum is turned on too quickly).

Aliquot

1. Label sterile 1.5 mL tubes with alginate code and number. Pre-weigh.
2. Aliquot and transfer dried alginate into tubes in the laminar flow hood by using sterile forceps. Promptly aliquot alginate from lyophilizer otherwise alginate will quickly absorb water from air.
 - a. Alternatively, remove the filter, and seal alginate within the 50 mL tube with a sterile cap.
3. Weigh, record difference. Goal is to have 5-10 mg per tube.
4. Store at -20°C until use.

Reconstitution

1. Allow tube to warm to room temperature.
2. Reconstitute with sterile solution in hood (1X PBS without Ca²⁺) to desired concentration.
3. Vortex briefly, then leave on orbital shaker overnight, or until completely dissolved.
4. Re-vortex tube aggressively then centrifuge briefly before use to remove air bubbles.

Calcium Solution

50 mM	CaCl ₂ (2.77 g)
140 mM	NaCl (4.1 g)
500 mL	MilliQ H ₂ O

- Measure 500 mL of MilliQ H₂O with a graduated cylinder
- Add CaCl₂ and NaCl to the water in a 500 mL beaker
- Mix with stir bar until thoroughly dissolved
- Sterile filter with a Millipore Stericup filter, in the hood
- Label with your name, the date, "Calcium Solution," and store at room temperature

Fetuin

(revised: June 25, 2007, MX)

Materials:

Fetal bovine fetuin
 High-quality MilliQ H₂O
 Sterile 50 mL conical tubes
 Clean 1.5 mL microcentrifuge tubes (puncture a hole in the lid with a large gauge needle)

Sterile Aliquoting:

- Fully dissolve Fetuin in MilliQ H₂O to a concentration of around ~15-20 mg/mL
- Thoroughly clean dialysis tubing with a large amount of water, and rinse with MilliQ water at least 10 times
- Fill the tubing with Fetuin solution, seal the tubing closed tightly, and dialyze in MilliQ H₂O (volume ratio=1:50 – 1:100) at 4°C overnight, stirring gently. Replace with fresh MilliQ H₂O, and dialyze 1-2 more times
- Measure the Fetuin concentration, using a BCA Protein Assay Reagent Kit
- Dilute Fetuin solution to desired concentration with MilliQ H₂O, usually 10 mg/mL
- Aliquot Fetuin into 50 mL conical tubes (40 mL/tube)
- Label tubes and freeze (1-2 days)
- Lyophilize to dryness (~5 days)
- Seal lids (place stickers over the holes) and store at -80°C for long-term storage or at -20°C for use within 6 months

Producing single aliquots (from 40 mL lyophilized aliquots, or from freshly dialyzed Fetuin)

- Add MilliQ H₂O to each tube to be aliquoted (10 mg/mL)
- Place conical tube on shaker until fully dissolved
- Aliquot fetuin into 1.5 mL microcentrifuge tubes (1 mL/tube)
- Close all of the tubes, and puncture a hole in the lid of each tube
- Freeze until completely solid (will take a few days at -20°C or overnight at -80°C)
- Transfer tubes to the lyophilizer as quickly as possible, and avoid letting the tubes thaw during this process. Keep tubes upright: placing tubes in cardboard holders from freezer boxes (cut smaller in size to fit in the chambers) will help
- Lyophilize to dryness (~1 day)
- Seal the holes in the tops of the lids with stickers, to keep your samples dry
- Store large amounts of aliquots in the -80°C freezer, but keep a box of tubes in the -20°C freezer for immediate use

Reconstituting:

- Reconstitute with α MEM to desired concentration
- Spin, vortex, and spin down again in centrifuge

Follicle Culture Media

Note: As with all medias, be sure to use clean glassware that has not been contaminated with harmful chemicals. Rinse all glassware carefully with MilliQ H₂O before media preparation to remove any bleach, detergent or contaminants. To avoid such issues, you may instead use 50 ml and 15 ml conical Falcon tubes. Prepare all media in the hood to keep stock solutions sterile. Media containing α MEM should be wrapped in foil for storage. Before use, warm Dissection media in a 37°C water bath for at least 15 minutes, but Maintenance, Growth, and Maturation medias must be equilibrated in petri dishes in the incubator (or in a conical tube with a loosened cap), for at least 30 minutes (α MEM based media needs to be kept in a 5% CO₂ environment to maintain it's pH at 7.3 or 7.4). Media should not be re-used after heating; it is best to aliquot the amount you need for the day, and warm it in the incubator/water bath, while placing the rest at 4°C for up to one week. Leftover Growth media may also be used as Maintenance media for future experiments. 3mg/mL BSA may be used to replace FBS (for DM and MM), but be sure to pH to 7.4 before using.

Dissection Media (DM, Dissociation, or L15):

30 mL	L15 (pre-made, stored at 4°C)
150 µL	Pen-Strep (pre-made, stored at 4°C)
300 µL	FBS (stock at -20°C; thaw and store at 4°C for up to 2 weeks)

- Invert gently to mix

Maintenance Media (MM):

30 mL	αMEM (pre-made, stored at 4°C) (add 300 µL L-glutamine if stock doesn't contain GlutaMax)
150 µL	Pen-Strep (pre-made, stored at 4°C)
300 µL	FBS (stock at -20°C; thaw and store at 4°C for up to 2 weeks)

- Invert gently to mix

Growth Media (GM):

30 mL	αMEM (pre-made, stored at 4°C; add 300 µL L-glutamine if stock doesn't contain GlutaMax)
30 mg	Fetuin (1 mg/ 1mL, stored at -20°C) <ul style="list-style-type: none"> • Reconstitute with desired amount of media • Add to media, rinse tube(s) into media
30 µL	ITS (1 µL/mL of 1000x stock, stored at -20°C, thaw and keep at 4°C for up to 2 weeks. *Be sure to label with your initials and date of thaw*)
90 mg	BSA (30mg/10mL, stored at 4°C)
300 mIU	rhFSH (final concentration 10mIU/mL)

- Place on a shaker for 3-5 minutes to ensure all components are mixed
- Spin down in clinical centrifuge for 3-5 minutes
- Sterile filter

Maturation Media (MatM):

9 mL	αMEM (pre-made, stored at 4°; add 100 µL L-glutamine if stock doesn't contain GlutaMax)
1 mL	FBS (stock at -20°C; thaw and store at 4°C for up to 2 weeks; 30 mg BSA may be used instead, but be sure to pH to 7.4)
1 µL	EGF (100 ng/µL stock, 10 ng/mL working)
15 µL	hCG (1 IU/µL stock; 1.5 IU/mL working)
0.45 uL	10 mIU/mL FSH (optional)

- Place on a shaker for 3-5 minutes to ensure all components are mixed
- Spin down in clinical centrifuge for 3-5 minutes (1500 RPM)
- Sterile filter *some prefer to add EGF, hCG, and FSH after filtering

FOLLICLE ISOLATION AND CULTURE FOR MICE

Follicular isolation takes about 30-60 minutes per animal (2 ovaries). Encapsulation only takes about 2 minutes for a few follicles. Plating beads usually takes less than 30 minutes. Image Capture and Analysis (day 0) takes up to 2.5 hours depending on yield.

For optimal results, all dissections are done in L15 media (buffered for open air use), on 37°C heated stages (temperature control), and on a clean bench (laminar flow hood) to minimize bacterial contamination.

Preparation

1. Prepare all of Dissecting Media (DM), Maintain Media (MM) and Growth Media (GM) according to the protocols contained within this handbook.
2. Place DM into the 37°C water bath (15-30 minutes to warm).
3. Place MM with loose cap into 5% CO₂ incubator for equilibration (30 minutes to warm).
4. Reconstitute alginate to desired concentration (See “Sterile Aliquoting of Alginate” on pg 3).
5. Sterilize mesh for Encapsulation (see “Follicle Encapsulation in Alginate” on pg 7).

Optional Enzymatic Isolation (best for animals 16+ days)

Materials for Enzymatic Media (EM):

DM (pg 4)

MM (pg 5)

Collagenase I (Stock: 10% in tubes of 20 µl and 50 µl, 0.1% working)

Dnase I (Stock: 2% in tubes of 20 µl and 50 µl, 0.02% working)

Procedure:

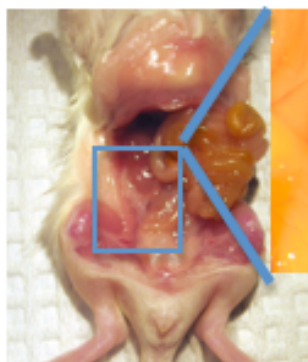
1. Thaw Collagenase and Dnase
2. Combine Collagenase and Dnase with MM to working concentration:
(20 µL Collagenase + 20 µL Dnase + 1900 µL MM)
(50 µL Collagenase + 50 µL Dnase + 4900 µL MM)
3. Gently mix the solution and sterile filter it into a new 35 mm dish. Place in the incubator to equilibrate while completing steps 1 and 2 of “Mechanical Follicle Isolation” below.
4. Place ovaries into Enzymatic Media (EM): cut larger ovaries in half before transferring them to EM. (Transfer ovaries with a wide-bore tip or by holding the ovary at the hillus with forceps – be careful to not squeeze the ovary with forceps)
5. Digest ovaries for 15-20 min (no more than 30 min) in EM in the 37°C, 5% CO₂ incubator (don't pipette ovaries up and down).
6. Rinse ovaries in clean DM, and then transfer them to a new dish of DM for isolation.
7. Proceed to follow the Mechanical Follicle Isolation procedure below, continuing with step 3.

Mechanical Follicle Isolation

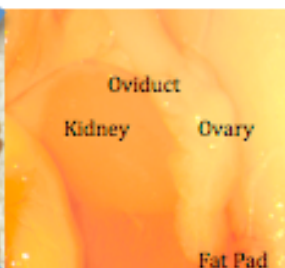
(See our video on the Oncofertility website: <http://oncofertility.northwestern.edu/media/secondary-follicle-isolation>)

(Revised October 2011, JP)

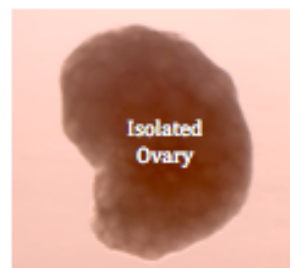
1. Euthanize 1 (or more) female(s) (day 12-16 best for follicle isolation), using CO₂ asphyxiation or isoflurane and a secondary method (cervical dislocation). Remove the ovaries from animal(s). In order to assure minimal damage to the ovaries, remove parts of the oviduct and uterus around the ovary as well. Place roughly dissected ovaries into a 35 mm dish of DM.
[Try to keep tissue out of the animal and out of an incubator for less than an hour. As a beginner, start isolating from one animal (2 ovaries) at a time, transferring follicles to MM and placing in the incubator before moving on to the next animal. Use a timer if it helps! As you become faster with isolation, you'll be able to isolate from more animals at a time.]
2. Under a dissecting scope, release the whole ovaries from uterus, fat pad and bursa by placing one pair of forceps at the intersection of the bursa and the oviduct to anchor the reproductive tract in place. Then place the second pair of forceps directly next to the first pair but only grip the thin membrane of the bursa. Carefully tear the bursa by gently pulling the two pairs of forceps apart, exposing the entire ovary. Transfer clean ovaries into a new 35 mm dish containing warm DM by grabbing the hilus region with forceps, or by pipetting them up with a wide-bore tip. Try to not squish the ovaries with your forceps.



Intact Reproductive Tract in Female Mouse



Ovary Encased Within Bursa and Surrounded by Reproductive Tract



Ovary Removed from Bursa and Surrounding Reproductive Tract

3. Isolate follicles from the first ovary by using two 28g½ Insulin syringes. With one insulin syringe in your non-dominant hand, anchor the ovary in your dish while using your dominant hand and second syringe to gently tease and “flick” individual follicles from the rest of the ovary. Try to remove as much stroma as possible without puncturing the follicle. Dissect out 20-40 secondary follicles per ovary (2 layer secondary: 100-130 µm, or multilayer secondary: 150-180 µm).
4. Add 1 ml MM in the central well, and 3 ml MM in the outer ring of an IVF dish. Transfer intact follicles to the outer ring of the IVF dish for a brief rinse and then selectively transfer them (see step 6) into the central well, placing no more than 60 follicles in the center of one IVF dish (so they won't all stick back together). Leave this IVF dish inside the incubator at 37°C, 5% CO₂.
5. Repeat step 1-5 on the remaining ovaries to finish all of the isolation.
6. After all follicles are in MM (1-2 hr), evaluate under 5x-8x magnification. Look for healthy follicles with the proper amount of granulosa cells; that are in the right size range; with no separation between oocyte and granulosa cells; and that have round oocytes. Some theca cells are fine. Separate “perfect” follicles into the center of your IVF dishes. **This step is THE KEY to a good experiment!!** These follicles will be encapsulated – keep multiple IVF dishes so you can rotate them in and out of the incubator.

Follicle Encapsulation in Alginate (mesh method)

(See our video on the Oncofertility website: <http://oncofertility.northwestern.edu/media/encapsulation-follicles-alginate-hydrogels>)

Materials

Isolated follicles, stored in MM in incubator (see above)

Alginate (pg 3)

Calcium Solution (pg 3)

MM (pg 5)

Polypropylene mesh squares (pg 17)

1. Cut polypropylene mesh into multiple 1" X 1" squares. Cover the mesh squares with 70% EtOH for roughly 30 minutes for sterilization, then place them into a clean 100 mm dish to dry within the laminar hood (~10 minutes) –OR– place mesh squares under UV light, overnight.
2. Load a 1mL syringe with your desired concentration of alginate and remove air bubbles.
3. Fill a 60mm dish about 2/3 of the way with RT calcium solution – set aside.
4. Create small droplets (~5 μ L each) through a 25G needle (1/2 in) onto the nylon mesh inside the lid of a 60 mm dish. Put 5-10 drops per mesh square.
5. Mouth pipette in an individual granulosa-oocyte complex (GOC) or follicle into each bead, transferring as little media as possible. If one group of follicles is transferred at the same time, make 1-2 extra alginate drops, and pipette all follicles into the first and then second “wash” drops to rinse; then transfer individual follicles into the other drops. Be sure to clear extra media out of your tip between washes, and replace it with a small amount of alginate before pulling follicles into your tip (to help eliminate media, and to help avoid air bubbles).
6. After all “non-wash” beads (0.5% alginate) have a follicle, lift the nylon mesh with forceps and turn it upside down over the calcium solution – hold it here for a few seconds. Flick mesh quickly to allow all beads to fall into the solution. Either pipette calcium solution on top of the beads or use forceps to push beads below the surface of the calcium solution, to ensure that the entire bead is submerged, and to allow for proper cross-linking. For 1.5% and 3% Alginate, submerge the entire mesh containing the alginate beads/follicles right side up into the calcium solution.
7. Crosslink the beads for the desired time (usually 2 min). Using forceps, gently transfer beads into a dish containing MM (no more than 20 beads per 35 mm dish). Keep the beads in the incubator until all encapsulation is complete.
8. Prepare 96-well plates for culture. To minimize the time that the plate spends outside of the incubator during imaging, a beginner should only put ~18-20 beads in each plate (1 bead/well). Place 100 μ L GM in each well to be used, and load surrounding wells with sterile 1X PBS. Leave plates in the incubator for at least 30 min to equilibrate before filling with beads.
9. Place 1 bead in each well containing GM and culture as desired.

Follicle Encapsulation in Alginate (Min’s alternative method)

Materials

Isolated follicles, stored in MM in incubator (see “Mechanical Follicle Isolation” pg 6-7)

Alginate (pg 3)

Calcium Solution (pg 3)

DM (pg 5)

MM (pg 5)

1. Prepare an IVF dish with 4-5 mL of calcium solution in the outer ring, and ~0.5 mL Alginate in the center ring (Alginate amount varies on the amount of follicles you have - figure that each follicle will require about 5 μ L of alginate)
2. Use your mouth pipette to transfer a few rounds of encapsulations' worth of follicles (~20) to DM. These follicles will be encapsulated within a very small amount of time, but it's better for them to be in DM while outside of the hood instead of MM (it will lose its pH quickly).
3. Use your mouth pipette to pick up 5 follicles from your dish of DM. Keep them separated in your tip by a small amount of media.
4. Gently place these follicles across the top of your alginate. Be careful to keep the follicles separated from one another, and work quickly so they won't fall through the alginate to the bottom of the dish - they'll be harder to pick up once they stick to the dish.
5. Using a 10 μ L pipette, set to 5 μ L, gently fill about half of the tip (~2.5 μ L) with alginate, pick up one of your follicles, then fill the rest of the tip (~2.5 μ L) with more alginate.
6. Slowly expel the alginate/follicle from the tip (perpendicular over the calcium solution), so it hangs like a drop from the end of the tip.
7. Either tap the hanging drop from the end of your tip, or very gently touch the very bottom of the drop to the calcium solution (do not touch your tip to the calcium as the remaining alginate inside of it will crosslink, causing it to clog).
8. Repeat steps 5-7 until all of the follicles in your IVF dish have been encapsulated.
9. Allow beads to crosslink for 2 minutes, then carefully transfer the beads with forceps to a new dish of MM.
10. Repeat steps 3-9 until all of the follicles from your DM have been encapsulated.
11. Repeat steps 2-10 until all of your isolated follicles have been encapsulated.

Tips for this method:

- Try to complete this entire encapsulation while keeping your alginate at RT. If you leave your alginate dish on a heating plate for too long, the PBS in it will evaporate, and cause your alginate to become thicker (and then it won't be 0.5% anymore!). Keep your follicles in either MM or DM at 37°C.
- Try using the same 10 μ L tip for multiple encapsulations – the only times you might need to switch to a new tip is when follicles start sticking to the alginate that remains on the outside of the tip; or if you dunk the tip in calcium solution, it may become clogged.

Follicle Encapsulation in Fibrin-Alginate IPN

Ariella Shikanov, Jan. 2009

Materials

Fibrinogen solution 50 mg/ml, aliquots of 200 μ L, stored at -20°C

Thrombin solution 500 mg(IU)/ml aliquots of 100 μ L, stored at -20°C

Alginate (ALG) solution 0.5%, or lower

TBS +Ca²⁺ 50 mM (sterile filter before using)

Glass slides with spacers and parafilm sprayed with 70% ethanol and dried in the hood

Isolated follicles, stored in MM in incubator (see “Mechanical Follicle Isolation” pg 6-7)

Procedure

1. Thaw Fibrinogen and Thrombin solutions.
2. Pipette the Thrombin solution up and down to mix it well and then dilute it 10-fold with TBS to obtain a concentration of 50 mg/ml.

3. After follicle isolation is complete, in a sterile tube, mix fibrinogen solution (50 mg/ml) and ALG 0.5% solution in a 1:1 ratio. **Caution!** Pipette fibrinogen solution very carefully to avoid forming bubbles. After the two solutions are combined, carefully pipette up and down several times (the mixture will turn from a clear to a milky solution), or vortex for a few seconds and then spin down.
4. Spot 7.5 µl drops of the Fibrinogen/ALG mixture on the parafilm coated glass slide (you can form up to 18 drops on 1 slide. The drops dry out pretty quickly, so for first-timers, start off with 5-6 drops at a time). To each drop, transfer 1 (or more) follicle(s) in a minimal amount of media (alternatively, you can “wash” the follicle in an additional drop of the mixture prior to final transfer).
5. Add 7.5 µl of Thrombin 50 mg/ml solution to each drop. Do not touch the follicle with the drop and do not mix. You don’t need to worry about the follicle sinking to the bottom.
6. Cover the drops with the second parafilm coated glass slide, put in a 100 mm dish upside down and transfer to the incubator for 5 min.
7. Transfer the beads to the Maintenance Media. The beads are sticky but not fragile, so transfer the beads by using tweezers.

Alternatively (according to Min Xu):

Mix fibrinogen, 0.5% alginate, and 1x PBS in a 1:1:2 ration. After transferring a follicle to the fibrinogen/ALG drop, Min sucks up the bead in a pipette tip and expels it into a petri dish containing **50 mg/ml** Thrombin solution. The crosslinking time is approximately 2 min or more (but not longer than 10 min).

Media Change (every other day)

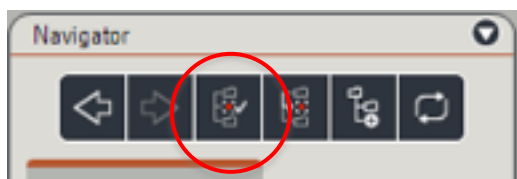
1. Aliquot the desired GM into a dish (50 µL/well), equilibrate it in the incubator until it is warm.
2. Image follicles (see “Follicle Image Collection” pg 9)
3. Using a multichannel pipette, carefully remove 50µl GM from each well and save (in a U-bottom 96 well plate, covered with a PCR plate sealer) at –80°C for future analysis. Add 50µl 1 pre-equilibrated GM into each well.
4. Do not keep plates out of incubator for more than 10 minutes at a time.

Imaging and Post-Culture Procedures

Follicle Image Collection

(Specific to Woodruff Lab imaging system: Leica Application Suite Version 3.6.0[Build:488])
(Revised October 2011, JP)

- Turn on Camera, Microscope Light Source and Heating Plate
- Set the microscope filter to match the objective you will be using (ex: HMC 20 for the Hoffman 20x objective)
- Open the Leica Application Suite imaging program
- Click on the [Browse] tab and set up the folder that you would like your images saved to. Be sure to set your capture location by highlighting the desired folder you would like your images to be saved to then pressing the [Set Capture Location] button.



- Click on the [Acquire] tab. This is where you will be able to view the live images and take pictures.
- Place 96-well plate on the stage, using the 20x HMC objective to locate your follicles. Using the coarse and fine focus knobs bring the follicle into focus then switch to the 4x HMC objective.
- Pay particular attention to focusing on the follicle's basement membrane.
- Press [Acquire Image] to take a picture. Images will automatically save to the folder that you initially set them to be stored to.

Note: Imaging should be completed as quickly as possible so that the time that follicles spend out of the incubator is kept to a minimum. This will reduce the chances that follicle health is affected by changes in media, pH, and temperature. If available, use of a warming stage set at 37°C is beneficial.

By taking pictures of 1-2 rows at a time then rotating to the next plate, you can avoid plates spending too much time outside of the incubator. Keep any plate(s) not being imaged in the incubator.

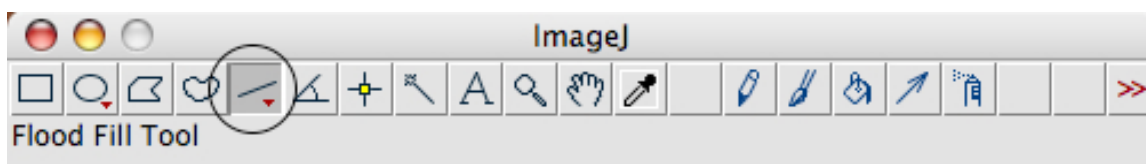
Measuring Follicle Diameter

- Open ImageJ (<http://rsbweb.nih.gov/ij/>)

Set your scale:

By a micrometer picture:

- Open your micrometer picture (taken at the same magnification as your pictures to be analyzed), by clicking File_Open and then by selecting the picture
- Click on the “Straight Line Selection” tool (which is the 5th tool from the left on the ImageJ tool bar)



- Draw a line on the picture, measuring a known distance
- While the line is still hi-lighted, click Analyze_SetScale...
- The “Set Scale” window will open. In this window, change the “Known Distance” field to the distance that you just measured with the straight line tool, and also change the “Unit of Length” to the corresponding unit. Be sure to click the “Global” box, to ensure that every picture you open while ImageJ is open will be measured with this scale. **Note:** Once you close ImageJ, you will have to re-set the Scale every time you re-open it.

By Pixels:

- Click Analyze_SetScale
- Set the “Distance in Pixels” and “Known Distance,” following the guide below, according to the magnification used to image your follicles:
 - 20X 208.2 pixels = 100 μ m
 - 10X 104.1 pixels = 100 μ m

- 5X 52.0 pixels = 100 μ m

Customize Outputs:

- Click Analyze > Set Measurements
- Select “Display Label,” so your image titles will appear in the measurement window
- Un-select any unnecessary measurements

Measure Follicles:

- To open images, go to File > Import > Image Sequence...
- Locate and open the proper folder, select the first image in the folder, and click “Open”
- The “Sequence Options” window will open – Select “OK”
- A window should then open containing all images within the folder. You can move between images using the scroll bar at the bottom, or by using the “<” and “>” keys on the keyboard
- Select the line tool (circled on pg 9)
- Draw a line across the widest diameter of the follicle
- Hit “m” to record the length of the line
- Draw a second line across the diameter of the follicle, perpendicular to the first line
- Hit “m” to record this length
- Lengths are recorded to the “Results” window
- When all measurements are complete, transfer values to excel by either:
 - Clicking Edit > Copy All in the “Results” window, and pasting the results into excel
 - Clicking File > Save As, and saving it as a “.xls” file

Isolating Follicles from Alginate Beads

Revised, SK 10/2009

Materials:

Alginate Lyase 10 IU/mL
DM or MM (pg 5)

Procedure:

1. Prepare alginate lyase solution (LS):
 - a. Add alginate lyase to media (10IU lyase/mL of media), pipette up and down a few times to mix, or invert. Allow LS to warm in incubator (MM based) or in a water bath (DM based) prior to use (for terminal procedures, dilute Lyase in DM: when continuing with cultures, dilute in MM). To lyase beads in the 96-well plate, prepare 100 μ L LS per well. To lyase beads together in an IVF dish, prepare 1mL LS per 15 follicles (1 dish).
2. Remove all media from wells containing beads, and add 100 μ L LS to each well.
 - a. Alternatively, remove 50 μ L of media from the wells (to freeze for media analysis), and use a blunt-tip or wide-bore tip to transfer the bead and remaining 50 μ L of media from the wells to be lysed to an IVF dish. Remove media from the IVF dish, and replace with 1 mL LS.
3. Incubate beads in LS on warming plate (DM-LS) or in the incubator (MM-LS) for 25-30 minutes.
4. Use a mouth pipette to remove follicles from plates (bend tip to gain access to small wells).

In Vitro Oocyte Maturation

Revised, SB 5/2008

Materials:

DM (pg 5)
MatM (pg 5)
Hyaluronidase
FHM, L15, or KSOM

Procedure:

1. Remove follicles from Alginate beads (see "Isolating follicles from alginate beads" on pg 10)
2. Prepare one IVF dish; add 3mL DM to the outer ring, and 1 mL to the inner ring. Transfer follicles from alginate beads (Step 4 "Isolating follicles from alginate beads") to the outer ring of your IVF dish, then to the inner ring (washing the follicles).
3. Prepare one IVF dish; which has 3 mL MatM) in the outer ring and 1 mL in the inner ring. (optional: cover inner ring with 200 μ L of oil)
5. Wash the follicles in MatM in the outer ring, and then transfer them into the inner ring. ***For large follicles, use a needle to nick the follicle to allow access of media to the COC. (Important step for non-antral follicles.)***
6. Incubate (37°C, 5% CO₂) for 15-16 hours (although it could take up to 20 hours)
7. To remove cumulus cells, add 10 μ L Hyaluronidase stock solution (final con. 0.3%); incubate (37°C, 5% CO₂) for 2-3mins. Mouth pipette to free oocytes from follicle. (This step is NOT necessary if you are going to fix the oocytes immediately, or are preparing for IVF. Use hyaluronidase if you are only imaging oocytes or grading rate of MII in your oocytes.)
8. Transfer oocytes to FHM, L15, or KSOM solution, and then examine oocyte stage (GV, MI, MII or Degenerated)

Fixation of Follicle/Alginate: 4% Paraformaldehyde (PFA)-Cacodylate-Ca²⁺ Fixation

(Revised Aug 28, 2006, MX)

Stock Solutions:

1. Reagent A (0.4 M Sodium Cacodylate Stock, pH=7.3-7.4):

C ₂ H ₆ AsO ₂ Na·3H ₂ O	8.56 g
Deionized H ₂ O	100 ml

Adjust pH=7.3~7.4 with 1N HCl, store at room temperature.

2. Reagent B (1M Sucrose):

Sucrose	3.423 g
H ₂ O	10 ml

Filter through 0.22 μ m, store at 4°C up to 1 month.

3. Reagent C (2M CaCl₂)

CaCl ₂ (anhydrous)	2.22 g
H ₂ O	10 ml

Filter through 0.22 μ m, store at room temperature.

Working Solution:

1. 4%PFA-Cacodylate-Ca²⁺ Fixation Buffer (4% PFA, 0.1M Sodium Cacodylate, 0.1M Sucrose, 10mM CaCl₂, pH=7.4)

- a. Weigh 1 g of PFA, add into 15 ml of ddH₂O, add 1-2 drops 1M NaOH

-scale this amount up or down depending on how much PFA you need

- b. Heat to 60°C and stir. **Make sure not to go above 60°C** as PFA breaks down into different components above that temperature. You will need to heat for about 20-30 minutes depending on how much PFA you are planning to make.
- c. After dissolving, turn the heat off, but continue to stir and let the solution cool to room temperature.
- d. Add 6.25 ml Reagent A, 2.5 ml Reagent B, 0.2 5ml Reagent C, mixture them and adjust pH=7.4. Then fill up to 25 mL with dH₂O in 50ml Falcon tube.
- e. Filter through a 0.22 µm syringe and use promptly. You can store Fixation Buffer at 4°C for up to 24 hours and use it again (re-filter prior to use). Otherwise throw it out and make fresh. The fresher the material, the better fixative it will be (especially for the ovary)

Fixation Procedure:

1. Remove all of culture media carefully, and add 100 µl 50mM CaCl₂/140mM NaCl to cross-link the alginate again for 2-3mins.
2. Usually, the bead will become more solid. Carefully take it out of the well by using Dumont forceps (type 7b, curved). Try your best not to squeeze the bead; otherwise it could destroy the antral follicle integrity.
3. Drop bead directly into 4%P FA-cacodylate-CaCl₂ fixation solution. Up to 20 beads can be put into 4mL fixation solution in a 5mL tube.
4. Lay down the tube and fix follicle for 4 hrs in 4 degree. Gently turn the tube up down every half hour. (*Note: Four hours fixation is enough. I haven't tried a shorter fixation time; it may also be ok.*)
5. Try to remove all of fixation solution carefully, without sucking the bead into tip. (*A small amount of fixation solution can be left inside of tube if it is too hard to remove it all.*)
6. Add 4 mL 1XPBS, and gently invert the tube a couple of times, then remove it quickly.
7. Repeat step 6.
8. Lightly dye the beads blue, to aid in post-fixation procedures (you'll be able to see the bead in the paraffin block).
 - a. Add 0.5% Alcian Blue/0.25% HAc stain solution; stain the bead inside for around 15 seconds, then take dye solution out ASAP. (*Don't leave the beads in dye solution for more than 1 minute: you DON'T want to stain the follicle!)(Stain solution can be reused)*)
9. Repeat steps 6 and 7
10. Ethanol Dehydration:
 - a. 50% EtOH for 10 min, repeat
 - b. 60% EtOH for 10 min, repeat
 - c. 70% EtOH for 10 min, repeat
 - d. The tissue can sit in 70% EtOH at 4°C.
11. However, do not let tissue sit in 70% EtOH for more than 2-3 days before processing.

*Alternatively, one may lyase beads (see "Isolating follicles from alginate beads" pg 11), and fix follicles in 1 mL of 4% PFA or Bouin's solution. Once fixed, briefly rinse follicles in PBS to remove excess fix, and then re-encapsulate all of the follicles in one group into one alginate bead (see "Follicle Encapsulation in Alginate" pg 7 or the alternative method on pg 8). It's best to also dye these beads with alcian blue, as described above, before transferring them straight to 70% ETOH.

Follicle Culture Certification

Getting Started

Lab members new to the follicle culture system must pass through a series of steps before becoming “certified” to culture follicles for experiments. Different steps will be easier or harder for every person. There is no set timeframe for how long this entire process will take, but we’ve found that a person with limited experience may pass through these steps and continue on to their own experiments within about 3 months (3 additional months may be required for the optional exercises). Don’t get stressed, ask as many questions as you can, and use your resources wisely! We have videos, protocols, and many lab members who already use this system that can help you out.

Objectives are loosely separated into different tiers, by degree of difficulty (with a large jump from Tier 3 to 4), but may be completed in any order. Please have a trained lab member (preferably your teacher, or Min) sign off after you have demonstrated your accomplishments to them. **These tasks are not to be completed as you are being taught, but instead as if YOU are the one teaching them – please be confident in your skill set before asking to check off the box you are working on.**

This sheet, along with your prepared packet must be presented to Teresa and Lonnie for review before initiating solo experiments.

TIER 1

- ☐ Gain access to the animal facility (the following 4 steps must be completed in order)
 - Complete online animal training
 - Complete Occupational Health Paperwork (and receive confirmation of clearance)
 - Assist in adding yourself to a Woodruff or Shea ACUC protocol
 - Complete the User Orientation Training course
 - For work with NHP or Human Tissue, please see Sarah K for additional safety and regulatory training information
- ☐ Demonstrate proper euthanasia methods
- ☐ Locate and remove intact ovaries of a pre-pubertal mouse
- ☐ Separate intact ovaries from the rest of the reproductive tract

TIER 2

- ☐ Assemble your own mouth pipette
- ☐ Prepare medias
 - Demonstrate sterile technique
 - Locate reagents
 - Demonstrate proper handling of common stocks such as FSH, ITS, alginate, EGF, HCG (how to thaw, label, store, where to store, etc).
 - Understand the requirements of properly labeling common and personal reagents (Name, Date of thaw), dispose of items when they expire
- ☐ Understand the basic principles of cell culture:
 - How is the pH of media maintained?
 - Which medias need CO₂, which ones do not?
 - What environmental factors could cause damage to follicles and gametes? How should you avoid them?
- ☐ Isolate 20 follicles (of any size) from 1 ovary

- ☐ Transfer 90% of isolated follicles from 1 dish to another, or from one drop to another via mouth pipette, losing less than 30% of starting follicles after 5 transfers.
- ☐ Encapsulate 50% of isolated follicles in alginate
- ☐ Demonstrate ability to identify freshly isolated follicles of different sizes with or without the use of an eyepiece micrometer

TIER 3

- ☐ Isolate 15 follicles of 1 size class from 1 ovary (choose the size class that you will most likely start using for your experiments)
 - 80-100 μm
 - 110-130 μm
 - 150-180 μm
- ☐ Encapsulate 80% of isolated follicles in alginate
- ☐ Image encapsulated follicles
 - Demonstrate competency with microscopy equipment (Leica DM IRB / Spot Advanced software)
 - Understand which lenses and filters to use in different situations
 - Capture clear images, with the proper amount of background
 - Demonstrate ability to image 12 wells within 10 minutes
- ☐ Demonstrate ability to properly use ImageJ (to measure follicle and oocyte diameter)
- ☐ Demonstrate ability to change media using multi-channel pipette with no more than a 5% rate of follicle loss

TIER 4

- ☐ Grow 40 follicles (150-180 μm) in culture for 8-9 days with >60% survival, and >40% antrum rate.
 - Fix follicles in alginate beads on day 8-9
 - Demonstrate knowledge of post-fixation processes and procedures to follow with the Histology core
 - Demonstrate ability to use Nikon Eclipse E600 microscope for imaging of sectioned, H&E stained follicles
- ☐ Grow 40 follicles (150-180 μm) in culture for 8-9 days with >60% survival
 - Lyase beads on day 8-9, and fix follicles separately (re-encapsulate follicles all in one bead for post-fixation procedures)
- ☐ Grow 40 follicles (150-180 μm) in culture for 8-9 days with >60% survival
 - Lyase beads, and perform IVM on day 8-9 (follicles should be >300 μm on day 8-9)
 - Recover 80% of the oocytes after IVM (regardless of their stages)
 - Demonstrate ability to distinguish MII, MI, GV and degenerated eggs (Optional)
 - Demonstrate >50% MII egg / survival follicle (Optional)
- ☐ Create % Survival Charts, and Growth Charts for 3 successful cultures (as signed off on above)
 - Demonstrate ability to use graphing software (such as Prism)

CERTIFICATION

- Prepare your package for review:
 - 3 Survival Charts (show them separately or on one graph)
 - 3 Growth Charts (show them separately or on one graph)
 - Bright field images of 1 follicle from multiple days in culture (d2, 4, 6, 8, 10)
 - One quality image of an H&E stained follicle from d8-9 of culture

- Any additional items you would like to include
- Meet with Teresa and Lonnie to discuss your certification
- Bring this sheet (all items must be signed off on)

Reagents/Supplies

Animals

The Woodruff lab uses CD1 mice for all follicle studies. Litters are sacrificed down to 12 pups before their 4th day of life, to cut down on nutritional variability between litters. These animals are housed in a temperature- and light-controlled environment (14L:10D) and are provided with food and water ad libidum. They are fed Teklad Global irradiated 2919 chow, which does not contain soybean or alfalfa meal and contains minimal phytoestrogens. Animals are treated in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the established Institutional Animal Care and Use Committee protocol at Northwestern University.

Follicle Culture Equipment/Supplies

Description	Company	Product #	Notes
0.22 um Millipore filters	Millipore	SLGP033RB	
1.7 mL microcentrifuge tube	Axygen	311-04-051	
50 mL Steriflip tube	Millipore	SCGP00525	
Conical Tubes, 15 mL	BD	352096	
Conical Tubes, 50 mL	BD	352070	
Culture plate, 96-well	Corning	3599	
Dish, Culture, 100 mm	BD	353003	
Dish, Culture, 35 mm	BD	353001	
Dish, Culture, 60 mm	BD	353002	
Dish, IVF	BD	353037	
Dissection Microscope (1)	Nikon	SMZ1500 with Binocular Eyepiece Tube for Diascopic Darkfield Use	Use with a heated stage
Dissection Microscope (2)	Leica	S8 APO with Leica TL BFDF	Use with a heated stage
Dumont #3c Forceps – Inox	Fine Science Tools	11231-20	
Dumont #7b Forceps – Inox Medical (curved)	Fine Science Tools	11270-20	
Forma Laminar Airflow Workstation	Thermo Scientific	1828	
Heated Stage for dissection scope	W. Nuhsbaum, Inc	STGWS2000L	Ask your local microscope supplier for a similar item
Heracell 150i CO ₂ Incubator	Thermo Scientific		

HSW Norm-Ject Syringes, Air-Tite, with Leur-Lock Tip	VWR	80076-428	Rubberless and free of silicone lubricants
Image J	NIH	http://rsbweb.nih.gov/ij/	
Insulin Syringe, 28G1/2	BD	329410	
Inverted Microscope	Leica	DM IRB	Use with a heated stage, ask your local supplier
Iris Scissors – Delicate Straight 10.5cm	Fine Science Tools	14060-10	
Leica Application Suite Version 3.6.0 [Build:488]	Leica Microsystems	http://www.leica-microsystems.com	Version 3.6.0 [Build:488]
Needle, 25G	BD	305122	
Premiere Slide Warmer	Premiere	XH-2001	
Spot Advanced	Diagnostic Instruments	Spot Advanced	
SPOT Insight 2MP Firewire Color Mosaic	Diagnostic Instruments	IN1820	Camera for inverted microscope
Surgical Scissors – Straight 14.5cm	Fine Science Tools	14002-14	
Syringe, 1mL	BD	309602	

Follicle Culture Reagent Info*

Description	Company	Catalog No.	Lot No.
Activated Charcoal	Fisher	05-690A	
Albumin Bovine (BSA)	MP Biomedicals	103700	S856J
Alcian Blue	Sigma-Aldrich	05500	
Alginate Lyase	Sigma	A1603	
Alginate**	NovaMatrix	4200101	
Ascorbic Acid	Sigma	A4403	
BCA Protein Assay Reagent Kit	Pierce	23227	
Bouin's Solution	Ricca Chemical Company	1120-32	
C ₂ H ₆ AsO ₂ Na. 3H ₂ O	Sigma	C-0250	
CaCl ₂	Wako	039-00475	SDF0564
Collagenase	Worthington Biochemical	CLS-1	
Dialysis Tubing	Spectra/Pro	132116, MWCO 8000	
DNase	Sigma	D4138	
Dulbecco's Phosphate Buffered Saline without Calcium Chloride & Magnesium Chloride, 1X	Gibco	14190	
EGF	Sigma	A412	
FBS	Gibco	10082-139	
Fetal Bovine Fetuin	Sigma	F-3385	094K7695
FHM w/out BSA (add BSA before use)	Chemicon	MR-122-D	

***Fibrinogen	Baxter/ Haemacure Corporation	1500952	
***FSH	Stock (gifted)	Alternative (Sigma F4021)	
HAc	Fisher	A38-212	
hCG	Sigma	CG-5	
HCl	Fisher	A38-212	
Hyaluronidase	Sigma	A1603	
ITS	Sigma	I1884-1VL	076K8913
KSOM w/ D-Glucose	Chemicon	MR-106-D	
L-15	Gibco	11415	
NaCl	Wako	191-01665	8497
Pen-Strep	Cellgro	30-002-CI	30002105
PFA	Fisher	T353-500	
Polypropylene Mesh	McMaster Carr	9275T21	
Sucrose	Sigma	S-1888	
Thrombin (Bovine)	Sigma	T-4648	
αMEM w/ Glutamax	Gibco	32561	

* Unless mentioned specially, all of reagents and media used should be at least cell-culture test, and embryo-tested grade is preferred.

** The alginate we are recommending for the culture system is from NovaMatrix, and it is PRONOVA UP MVG (#4200101). The company produces a range of alginates, which are given in the following link (http://www.novamatrix.biz/Portals/novamatrix/Content/Docs/ProductDocs/alginate_General_info.pdf). There are approved medical devices currently marketed that contain our alginates and many more in clinical development. The cited alginate is the closest to the alginate used in the majority of our published studies. We have cultured follicles in this alginate and found that they do grow well, but the percentages do not correlate exactly with the mechanical properties. In other words, while we often use a 0.25% solution of our alginate to create a permissive environment for follicles, the percentage of alginate to create this same mechanical environment using the new alginate is ~0.15%. We notes that their MVG alginate is a low endotoxin alginate, high G/M raitio that can be made in a range of molecular weights. They have established safety and toxicity data on these purified alginates and have filed drug master and device master files with the FDA to support drug and device filings by our partners and customers.

***Gifted: Fibrin Sealant Kit(Hemaseel APR Kit from Haemacure Corp), Fibrinogen from Calbiochem (500gm) Cat#341578 is also another option.

****Serenio, recombinant hFSH

Mouth Pipette Construction

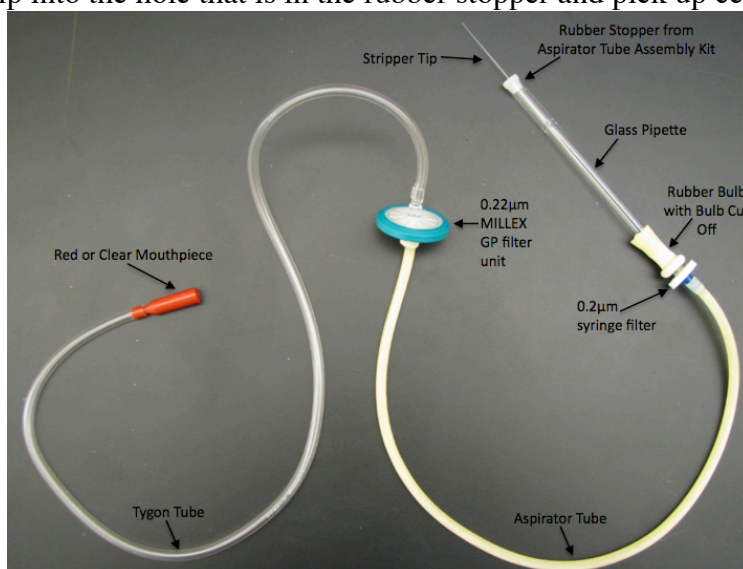
*****Mouth pipetting should only be used when using mouse tissue. If you plan on using non-rodent tissue (i.e. non-human primate, bovine, human, etc.), mouth pipetting is NOT allowed. In this case, a stripper pipette and tips should be used (Cat. # MXL3-STR). This is to protect the tissue from the user and to protect the user from any fecal/ intestinal bacterial contamination, infectious diseases, and blood borne pathogens.**

Description	Company	Product #	Notes
0.2 um Syringe Filter	Nalgene	180-1320	
Aspirator tube assemblies for calibrated microcapillary pipettes	Sigma-Aldrich	A5177-5EA	
Glass Pipette	VWR	14672-200	5 ¾ inch flint glass

MILLEX GP filter unit 0.22 μ m	MILLIPORE	SLGP033RB	
Rubber Bulb 1mL	Fisher Scientific	03-448-21	
Silicone Tips for Bulb Assembly	VWR	53507-275	
Stripper Tips: 70 μ m 100 μ m 150 μ m 200 μ m 275 μ m 600 μ m	Mid Atlantic	MXL3-203-70 MXL3-203-100 MXL3-203-150 MXL3-203-200 MXL3-203-275 MXL3-203-600	
TYGON Application Specific Tubing from Saint-Gobain Performance Plastic	Fisher Scientific	AAC00006	TYG tub 1/8 IN x 3/16 IN Length: 50ft Wall: 1/32 IN

To make a mouth pipette:

- 1) Detach the mouth piece and rubber stopper from the aspirator tube
- 2) Cut a piece of TYGON tubing (clear tube) into a 17 inch section
- 3) Place the small end of the mouth piece into one open side of the TYGON tube
- 4) Into the other end of the 17-inch TYGON tubing, put the tapered, smaller end of the 0.22 μ m MILLEX GP Filter
- 5) Around the wider projection on the other side of the 0.22 MILLEX filter, securely place it into one open end of the aspirator tube (yellowish in color)
- 6) Then take the small, 0.2 μ m Syringe Filter and insert the tapered smaller end into the other side of the aspirator tube
- 7) Cut the top bulbous portion off of the rubber bulb and wrap the tight, lipped, non-cut end around the wider projection on the other side of the 0.2 μ m Syringe Filter
- 8) To make the glass pipette body, it is necessary to remove the tip. To do so, wearing a pair of goggles, carefully break off the top narrow portion at the point where it widens. Melt down the sharp edges with a Bunsen burner flame.
- 9) After cooling, place the melted down end of the glass pipette into the cut end of the rubber bulb
- 10) In the other end of the glass pipette place the rubber stopper from the aspirator tube assembly kit, tapered side down
- 11) Insert the stripper tip into the hole that is in the rubber stopper and pick up cells as needed



References

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Please also see a more detailed list of our publications on the Oncofertility website:

<http://oncofertility.northwestern.edu/researchers/selected-oncofertility-publications>

<http://oncofertility.northwestern.edu/researchers/oncofertility-publications-mouse-follicle-culture>