King Lab Choanoflagellate Protocol Handbook

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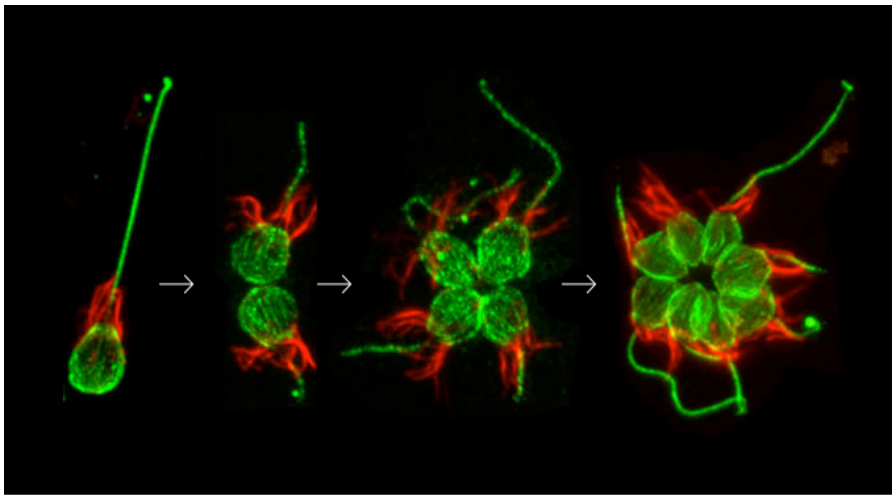

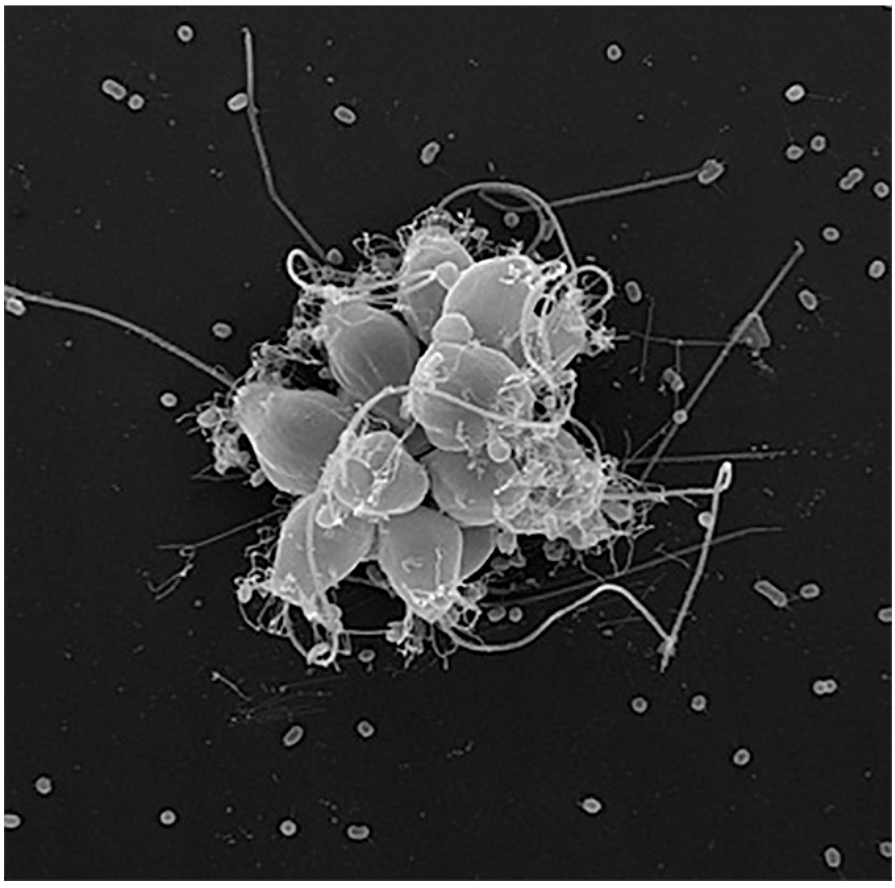

Welcome to the study of choanoflagellates!

This handbook is provided to help you get started culturing choanos in your own lab and aims to provide the most common protocols used within the King lab at the University of California, Berkeley. These protocols are geared towards the current strain of focus in our lab, the monoxenic culture *Salpingoeca rosetta* (ATCC PRA-390). In addition, these protocols are optimized for molecular biology protocols. Some reagents and procedures may be modified for general choanoflagellate husbandry purposes.

Other strains available from ATCC (atcc.org) are:

- *Monosiga brevicollis* (ATCC 50154)
- *Monosiga brevicollis* MX1, sequenced for the genome project (ATCC PRA-258)
- *Monosiga ovata* (ATCC 50635)
- S*alpingoeca rosetta* (formerly known as Proterospongia sp. ATCC 50818)
- *Salpingoeca rosetta* Px1, sequenced for the genome project (ATCC PRA-366)
- *Helgoeca nana* (ATCC 50073)

The bacteria required for rosette induction in *S. rosetta* are also available from ATCC: *Algoriphagus machipongonensis* (ATCC BAA-2233).

cDNA libraries from *Monosiga brevicollis* (#25864) and *Salpingoeca rosetta* (#62843 & #62844) have been prepared and are available at Addgene (www.addgene.org)

Various additional protocols have been published and are available in the Cold Spring Harbors Protocols Manual and can be found at http://cshprotocols.cshlp.org/content/2009/2/pdb.emo116.abstract

We hope you come to love choanoflagellates as much as we do. Good luck with your research!

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Common Media and Solutions

Any media can be made into an agar plate by adding 8g agar (Bacto cat#214050) per 400ml of media, autoclaving on a liquid cycle for 20 minutes and pouring 20mls into each petri dish (Fisher cat# FB0875713)

Standard Growth Conditions

Strain	ATCC	Media
SrEpac	PRA-390	5% SWC diluted with 100% ASW
PX1	PRA 366	25% CGM diluted with 100% ASW
RCA	50818	10% CGM diluted with 100% ASW

Artificial Seawater (ASW)

Artificial Seawater is a minimal nutrient medium forms the basis of other media (e.g. CGM3 and SWC) used for culturing choanoflagellates. In addition, it is used at 100% strength for starving choanoflagellates.

For 2 Liters of ASW

$7.9 < pH < 8.1$

Materials

- Sterile 4L Erlenmeyer Flask
- Graduated cylinder
- Tropic Marin Sea Salt (www. tropic-marin.com, we use catalog # 10134)
- Magnetic stir bar
- MilliQ Water

Procedure

1. Weigh out 65.8g Tropic Marin Sea Salt, add to a 4L flask along with a stir bar
2. Add 1600mL of ultrapure water to the sea salt and stir until the salts are mostly dissolved, some salt particles will still be visible
3. Adjust pH with HCl to between 7.9-8.1
4. Add MilliQ water to bring volume to 2L and stir for ~5 min
5. Aliquot as needed into desired volumes
6. Autoclave on liquid cycle for 30 minutes to sterilize

7. Store at room temperature.

Seawater Complete Media (SWC)

Seawater Complete Media is a growth medium used to culture various strains of choanoflagellates.

For 1 Liter of media:

Materials

- Sterile 2L Erlenmeyer Flask
- Graduated cylinder
- Magnetic stir bar
- Tropic Marin Sea Salt (www. tropic-marin.com, we use catalog # 10134)
- 50% glycerol
- MiilliQ water
- Bacto Peptone (catalog #211677)
- Bacto Yeast extract (catalog #210933)
- 0.22µm sterile filter (Millipore catalog # SCGPU05RE)

Procedure

1. Weigh out 24g Tropic Marin Sea Salt, 5g peptone and 3g yeast extract and add to a 2L flask along with a stir bar.
2. Add 6mL 50% glycerol
3. Add 1600 mL of MilliQ water to the sea salt and stir until the salts are mostly dissolved, some salt particles will still be visible
4. Add ultrapure water to bring to a final volume of 2L and stir for ~5 min
5. Aliquot as needed into desired volumes
6. Autoclave on liquid cycle for 30 minutes to sterilize
7. Filter sterilize with 0.22µm filter and vacuum line
8. Store at room temperature.

Cereal Grass Media (CGM)

Cereal Grass Media is used as a growth medium for PX1 and is typically prepared using hot Artificial Seawater directly from the autoclave since it requires 3-3.5 hours of "brewing time." Along with standard lab glassware, you will need a large Buchner funnel fitted with a rubber stopper.

For 1 Liter of media
$7.9 < pH < 8.1$

Materials

- 1L Artificial Seawater (ASW) directly from autoclave
- Graduated cylinder
- Sterile 2L Erlenmeyer flask
- Sterile 2L Buchner flask
- Magnetic stir bar
- Cereal Grass (Basic Science Supplies catalog # IS5020)
- Buchner Funnel (Coorstek catalog # 60246)
- #1 Whatman paper (catalog # 1001-150)
- 0.22-micron sterile filters (Millipore catalog # SCGPU05RE)

Procedure

1. Use hot ASW directly from autoclave
2. Add Cereal Grass to the media (5g / L).
3. Allow the media to steep for 3 – 3.5 hours.
4. Rough filtration: Use a sterile 2L Buchner flask. Pre-wet 3 pieces of Whatman 150mm filter paper with ddH2O and place in Buchner funnel. Vacuum filter media to remove large particles. Repeat this filter process a 2nd time with fresh filter paper. Discard (or compost!) cereal grass particles and filter paper
5. Aliquot into desired volumes and filter sterilize with 0.22µm filter and vacuum line
6. Store at room temperature.

25% Freshwater Cereal Grass Medium (FCGM)

Freshwater cereal grass medium is used to culture some freshwater species of choanoflagellates.

For 2 Liter of media

Materials

- 4L sterile Erlenmeyer Flask
- Sterile 4L Buchner flask
- Ultrapure Water
- Cereal Grass (Basic Science Supplies catalog # IS5020)
- Buchner Funnel (Coorstek catalog # 60246)
- #1 Whatman paper (catalog # 1001-150)
- 0.22-micron sterile filters (Millipore catalog # SCGPU05RE)

Procedure

1. Add 2L of ultrapure water into a 4L sterile Erlenmeyer flask.
2. Boil water by autoclaving for 40 min on liquid cycle
3. Add 2.5g Cereal Grass to boiling MilliQ water
4. Allow the media to steep for 3-3.5 hours at room temperature
5. Rough filtration: Use a sterile 2L filtering Erlenmeyer flask. Pre-wet 3 pieces of Whatman 150mm filter paper with ddH2O and place in Buchner funnel. Vacuum filter media to remove large particles. Repeat this filter process a 2nd time with fresh filter paper. Discard cereal grass particles and filter paper
6. Aliquot into desired volumes and filter sterilize with 0.22µm filter and vacuum line. This will go slowly and the filter may clog, requiring you to switch to a new filter.
7. Store at room temperature

Water Complete Cereal Grass Medium (WC-CGM)

WC-CGM medium is used to culture some freshwater species of choanoflagellates.

For 2 Liters of media

Materials
Stock solutions [g/1000 ml dist. H_2O] Can be made in smaller volumes. Store at RT.

- $CaCl_2 \cdot 2H_2O$ (36.8g) CAS 10035-04-8
- $MgSO_4 \cdot 7H_2O$ (37g) CAS 10034-99-8
- $NaHCO_3$ (12.6g) CAS 144-55-8
- $K_2HPO_4 \cdot 3H_2O$ (11.4g) CAS 26788-57-1
- $NaNO_3$ (85g) CAS 7631-99-4
- $Na_2SiO_3 \cdot 9H2O$ (21.2 g) CAS 13517-24-3
- Triethylsilane powder (TES) (0.115g) CAS 617-86-7

Micronutrient solution [g/1000 ml dist. H2O] Store at 4°C

- Na_2EDTA (4.36g) CAS 6381-92-6
- $FeCl_3 \cdot 6H_2O$ (3.15g) CAS 10025-77-1
- $CuSO_4 \cdot 5H_2O$ (0.01g) CAS 7758-99-8
- $ZnSO_4 \cdot H_2O$ (0.022g) CAS 7446-19-7
- $CoCl_2 \cdot 6H_2O$ (0.01g) CAS 7791-13-1
- $MnCl_2 \cdot 4H_2O$ (0.18g) CAS 13446-34-9
- $Na_2MoO_4 \cdot 2H_2O$ (0.006g) CAS 10102-40-6
- H_3BO_3 (1g) CAS 10043-35-3

Vitamin solution [g/1000 ml dist. H2O] Store at 4°C

- thiamine HCl (0.1g) CAS 67-03-8
- biotin (0.0005g) CAS 58-85-5

Procedure

1. Prepare each of the stock solutions and add 2ml of each to 2 liters of distilled water in a sterile 4L Erlenmeyer flask
2. Autoclave for 40 min.
3. Add 10g Cereal Grass to boiling solution to make 100% WC-CGM3.
4. Allow media to steep ~3-3.5 hours
5. Rough filtration: Use a sterile 2L filtering Erlenmeyer flask. Pre-wet 2 pieces of Whatman 150mm filter paper with ddH2O and place in Buchner funnel. Vacuum filter media to remove large particles. Repeat this filter process a

second time with fresh filter paper. Discard cereal grass particles and filter paper

6. Aliquot into desired volumes and filter sterilize with 0.22μm filter and vacuum line
7. Store at room temperature

Starting cultures from a frozen stock

This protocol describes how to thaw choanoflagellate cultures.

NOTE: Media are diluted in ASW to the indicated percentage (v/v)

Materials

- Seawater Complete (SWC)
- Artificial Seawater (ASW)
- Cereal Grass Media (CGM)
- *S. rosetta* stock in freezer vial (ATCC strains: PRA-390, PRA 366, or 50818)
- 5 or 10 mL sterile pipette (glass or disposable)
- Cell lifter (cat. no. 07-200-365, Corning)
- Vented cap uncoated 25 cm tissue culture flask (e.g. Corning Standard 25 cm Vented Cap Tissue Flask, cat. no. 430639)
- Tissue culture hood

Procedure

NOTE: To avoid contamination with new and unwanted species of bacteria, all steps should be performed using sterile technique in a tissue culture hood if possible.

1. In a standard 25cm tissue culture flask add 9 mL of media for the indicated strain

Strain	ATCC	Media
SrEpac	PRA-390	1% SWC
PX1	PRA 366	10% CGM
RCA	50818	10% CGM

2. Starting with a vial of frozen cells, thaw cells in hand or at room temperature.
3. When thawed, immediately and gently pipette 1 mL of cells into 9 mL of indicated media. Incubate culture either in a 25°C incubator or at room temperature. The culture may initially look overgrown with bacteria, but within 2-6 days the choanoflagellates should out-compete their bacterial food. Once the choanoflagellates have reached a cell density of (10^6 – 10^7 cells/ mL), you will need to split them into fresh media (see below).

Determining Cell Density

Materials

- 37% formaldehyde
- Hemocytometer (Fisher catalog# 02-671-6)
- Inverted lens microscope (e.g. Leica DMIL equipped with 20x and 40x objectives)

1. Pipette 200 ul of cells from the culture you wish to count in eppendorf tube.
2. Add 1 ul of 37% formaldehyde to cells and mix thoroughly by vortexing.
3. Add 10 ul of formaldehyde fixed cells to hemocytometer.
4. Count cells and apply appropriate conversion factor according to hemocytometer manufacturer's directions.

Maintaining and sub-culturing cultures

These protocols are to help maintain and expand *S. rosetta* cultures in preparation for DNA or RNA isolation or cell biological assays. *S. rosetta* is co-cultured with bacteria as a food source.

Strain	ATCC	Media
SrEpac	PRA-390	5% SWC
PX1	PRA 366	25% CGM
RCA	50818	10% CGM

RCA: *S. rosetta* + mixed environmental bacteria (ATCC 50818)

1. Start with a culture of *S. rosetta* at maximum density (10^6 – 10^7 cells/ mL), typically seeded from a frozen stock.

2. Using the cell lifter, scrape the bottom of the dish to re-suspend any adherent cells.

3. Add 1 mL of cells to 9 mL 10% CGM. Incubate culture in a 22°C incubator or at room temperature.

4. Monitor growth and viability of cells on an inverted lens microscope at 200 – 400x magnification.

5. Split culture into fresh 10% CGM every 2 – 4 days.

SrEpac: *S. rosetta* + *Echinicola pacifica* (ATCC PRA-390)

1. Start with a culture of *S. rosetta* at maximum density (10^6 – 10^7 cells/ mL), typically seeded from a frozen stock.

2. Add 1 mL of cells to 9 mL 5% SWC. Incubate culture in a 22°C incubator or at room temperature.

3. Monitor growth and viability of cells on an inverted lens microscope at 200 – 400x magnification.

4. Split culture into fresh 5% SWC every 2 – 3 days.

PX1: *S. rosetta* + *Algoriphagus machipongonensis* (ATCC PRA-366)

1. Start with a culture of *S. rosetta* at maximum density (10^6 – 10^7 cells/ mL), typically seeded from a frozen stock.

2. Add 2 mL of cells to 8 mL 25% CGM. Incubate culture in a 22°C incubator or at room temperature.

3. Monitor growth and viability of cells on an inverted lens microscope at 200 – 400x magnification.

4. Split culture into fresh 25% CGM every 2 – 4 days.

Troubleshooting cell culturing

These are some common issues that can arise when culturing choanoflagellates and the recommended solutions.

1. Problem: Overgrowth of co-cultured bacteria

   Solution: Bacterial growth is a fact of life in choanoflagellate cultures. If bacteria begin to dominate the culture and interfere with choanoflagellate proliferation, start over with a freshly thawed stock of cells. *S. rosetta* has approximately a 6 hour doubling time during log phase and should reach a maximal density of 10^6 – 10^7 cells/ mL.

2. Problem: The expected cell type is not present in cultures (e.g. no rosettes are present in PX1)

   Solution: Continue passaging supernatant until cell type emerges to re-equilibrate the bacterial choanoflagellate ecosystem.

3. Problem: A lot of bacterial biofilms are present in the culture

   Solution: Place flask upright for several hours to allow biofilms and clumps to settle to the bottom, then only passage cells from the very top of the culture into fresh media.

Long-Term Frozen Storage

Choanoflagellates can be stored under stable conditions by freezing them with liquid nitrogen, as described here. To prevent cross-contamination among vials stored in the liquid phase of a liquid nitrogen freezer, the use of CryoFlex tubing to fully seal the vials is encouraged.

Materials

- Culture of choanoflagellates grown to maximal density (10^6-10^7 cells/mL)
- Dimethyl sulfoxide "DMSO" (Sigma catalog # 472301)
- Isopropanol
- Bunsen burner
- Dewar storage rack
- Freezing container, rate-controlled (e.g., "Mr. Frosty" Cryo 1°C Freezing Container; Nalgene 5100-0001)
- Liquid nitrogen
- Pliers, needle-nose
- Scissors
- Tubing, CryoFlex (Nunc 343958)
- Vials, CryoTube, screw-capped, internally threaded, 1-mL (Nunc 366656)

Procedure

1. Precut the CryoFlex tubing into 6.5-cm lengths with scissors.

2. Add 900 μ L of choanoflagellate cells and 100 μ L of DMSO to a CryoTube vial. Invert the tube repeatedly to mix.

3. Slide the vial into a precut tubing section. Quickly pass the covered vial through the flame of a Bunsen burner so that the tube shrinks around the vial. Crimp the ends with a pair of needle-nose pliers.

4. Freeze the covered vial overnight at -80°C in a rate-controlled freezer filled with isopropanol.

5. The next day, transfer the frozen vial to a Dewar storage rack containing liquid nitrogen for long-term storage.

NOTE: Make sure to transfer frozen cells to liquid nitrogen after 24 h. Choanoflagellates cannot be stored stably at -80°C and might not recover from freezing if stored at this temperature.

Culturing *Algoriphagus machipongonensis*

The bacterium *Algoriphagus machipongonensis* (ATCC BAA-2233) produces lipids that induce rosette development in *S. rosetta*. We usually start with a culture of SrEpac ATCC PRA-390 (*S. rosetta* + *E. pacifica*, as this culture typically produces rosettes at higher rates than other cultures.

Culturing *Algoriphagus machipongonensis*

Materials

- Glycerol stock of *Algoriphagus machipongonensis* (ATCC BAA-2233)
- 100% SWC agar plate
- 100% SWC liquid media

Procedure

1. Streak out *Algoriphagus machipongonensis* from glycerol stock onto 100% SWC agar plate. Allow plate to grow at room temperature (22°C-25°C). It may take about 2-3 days for colonies to be visible.

2. Inoculate *Algoriphagus machipongonensis* colony into desired volume of 100% SWC media. Grow shaking at 30°C for 24-48 hours

3. When culture OD_{600}nm is between 0.250-0.300, the culture is ready to be used for rosette induction.

Rosette induction

This protocol describes the methods to induce rosette formation in the otherwise single-celled SrEpac.

Materials

- Culture of *Algoriphagus machipongonensis* at OD_{600}nm 0.250-0.300
- Culture of S. rosetta + E.pacifica (ATCC PRA-390)

Procedure

1. Add 50uL *Algoriphagus* liquid culture into 10mL of recently split *S. rosetta* + *E. pacifica* culture.

2. Continue to grow *S. rosetta* culture. Rosette induction can take anywhere between 18-48 hours depending on the initial growth rate of *S. rosetta.*

S. rosetta Immunofluorescence Protocol

Materials

- Round poly-L-lysine coated cover slips (Corning catalog # 354087)
- 24-well tissue culture plate (Corning catalog # 3526)
- Fix Solution: Fresh 4% Paraformaldehyde/Formaldehyde diluted in 4x PBS
- Block: 1% BSA; 0.3% Triton; PEM
- PEM (100 mM PIPES pH 6.9, 1 mM EGTA, 1 mM $MgSO_4$)
- BSA
- Triton X-100
- Fresh 6% acetone diluted in 4x PBS
- Appropriate primary and secondary antibodies
- Hoechst 33342 (Life Technologies catalog # H3570)
- Rhodamine Phalloidin (Life Technologies catalog # R415)
- Pro-Long Gold (Life Technologies catalog # P37930)
- Microscope slides
- Clear nail polish

*NOTE: Antibodies should be diluted according to their individual instructions and vary by antibody

Fix Solution: 4% Paraformaldehyde in 4x PBS (200uL per cover slip)

25 uL	32% Paraformaldehyde
40 uL	20x PBS
135 uL	dH_2O

6% Acetone in 4x PBS (200uL per cover slip)

12 uL	100% Acetone
40 uL	20x PBS
148 uL	dH2O

Note: Dilute in 1x PBS for both the 4% Paraformaldehyde and 6% Acetone solutions when working with fresh water species.

Block Solution (1% BSA, 0.3% Triton, PEM) for 10mL:

0.1g	BSA
300 uL	10% Triton X-100
9.7 mL	PEM

PEM Solution (100 mM PIPES pH 6.9, 1 mM EGTA, 1 mM $MgSO_4$)

1. Add . Add 800 ml of dH2O to a 2L Erlenmeyer flask along with a stir bar.

2. Add 30.24g of PIPES to the solution. (0.1M x 1L x 302.37g/mole = 30.237g)

3. Add 2 ml of 0.5M MgS04 using a pipette to the solution. (0.5M x V = 0.001M x 1L) V= 0.002L or 2 ml

4. Add 0.38g of EGTA (0.001M x 1L x 380.35g/mole = 0.38g)

5. Add approximately 2 ml of 10 M NaOH to fully bring all solids into solution.

6. Calibrate the pH meter and adjust the final pH to 6.9.

7. Adjust the final volume to 1L by adding dH2O.

8. Sterifilter the PEM solution with a 0.22 micron filter and aliquot into 500ml flasks.

9. Store at 4 degrees Celsius in the refrigerator until ready to use.

Procedure

1. For each sample, place one round poly-L-lysine coated cover slip per well in a 24 well plate.

2. Add 2 mL of culture to each well containing a coverslip. Incubate for 30 min at room temperature to allow cells to settle onto coverslip. (Note: Some cell types require an additional spin to pellet cells onto the coverslip however, this can disrupt chain morphology)

3. Gently remove almost all media leaving a thin layer covering the coverslip. (Note: Always leave a small layer of solution covering the coverslip so the cells don't dry out)

4. Gently add 200µL of 6% Acetone diluted with 4x PBS for 5 min. at room temperature. (Note: Always use a P200 pipette to reduce the chance of losing cells due to force from pipetting. Also, pipette buffers onto the side of the well, and always remove buffers on the side).

5. Remove 6% Acetone, and add 200µL of 4% of fix solution. Incubate at room temperature for 15 min.

6. Gently remove fix solution and add 200µL of PEM to wash.

7. Pipette off Pem solution and add 200ul of block solution to block and permeabilize the sample. Incubate for 30 min.

8. Remove block and add 200µL of 1° antibody diluted in block. Incubate for 1 hour at RT.

9. Remove 1° antibody solution and add 200µL of PEM to wash. Repeat 2 more times for a total of 3 washes.

10. Remove PEM solution and add 200µL of 2° antibody diluted in block. Incubate for 1 hour in the dark at RT.

11. Remove 2˚ antibody solution and add 200uL of block to wash sample. Repeat one additional time. Wash 2x in 200µL Block

12. Remove block and add 200uL of PEM to wash.

13. Remove PEM and add 200 uL of Hoechst 33342 diluted 1:1000 and add Rhodamine Phalloidin diluted 1:20 in PEM. Incubate at RT for 30 min.

14. Remove Hoechst, Rhodamine and PEM solution and add 200µL of PEM to wash.

15. Prepare slide by adding 10µl of Pro-Long Gold in a drop where you will place the coverslip.

16. With tweezers, gently remove the coverslip, briefly dab the edge of the coverslip onto a kimwipe to catch excess PEM, and carefully place onto the drop of Pro-Long Gold.

17. Incubate in dark for 30 min to O/N to allow Pro-Long Gold to set.

18. Seal edges of coverslip with nail polish and image.