IMMUNOCYTOCHEMISRY OF INTACT FOLLICLES

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Whole mount immunofluorescence of mouse ovarian follicles

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Adapted from protocols by Pam Kreeger and Susan Barrett

This procedure works best with primary and two layer secondary follicles. Multilayer follicles will fix and stain but can be difficult to image well. There are two types of fixation given: 2% formaldehyde and 4% paraformaldehyde. The formaldehyde with Stabilization buffer is the preferred fixation method if detailed cytoskeletal information is desired. Paraformaldehyde in PBS is sufficient for all other proteins.

Materials

5X stabilization buffer (SB), 50 mL

	Add	**Final conc.**
PIPES*	7.550 g	500 mM
$MgCl_2$-6H_2O	0.250 g	25 mM
EGTA	0.235 g	12 mM
ddH_2O	50 mL	-

*Note: PIPES will only dissolve between pH 6.1-7.5.
Filter solution and store at 4°C for up to one year.

Fix (choose one):

a.) 4% paraformaldehyde, 1 mL

	Add	**Final Conc.**
16% formaldehyde (in PBS)	250 µL	4%
ddH2O	750 µL	-

b.) 2% formaldehyde, 1 mL

	Add	**Final Conc.**
SB	200 µL	-
37% formaldehyde (v/v)	54 µL	2% (v/v)
Triton X-100	20 µL	2% (v/v)
ddH_2O	726 µL	-

Blocking/Washing Buffer

	Add	**Final Conc.**
1X PBS	100 mL	-
NaN_3	200 mg	0.2% (w/v)
Skim milk*	200 mg	0.2% (w/v)
Normal goat serum*	2 mL	2% (v/v)
BSA*	1 g	1% (w/v)
Glycine**	750 mg	0.1 M
Triton X-100	100 µL	0.1% (v/v)

Filter solution and store at 4°C.
*Binds to IgGs (block). **Reacts with free aldehyde groups.

Methods

Fixation

1. Warm fixative in dish to 37°C before use. You can use any container for fixation (e.g. IVF dish, four-well dish, microwell dish, etc.)
2. Transfer follicles with a minimal volume of medium to the fixation dish. (You may wish to remove excess media by transferring follicles into 1X PBS before adding to dish with fixative.)
3. Fix follicles for 45 min to 1 hour at 37°C with shaking. (45 minutes for smaller follicles, 1 hour for larger).
4. Wash follicles in blocking/washing buffer at least three times, 10 mins each.

Blocking

5. Incubate follicles in blocking/washing buffer for 1 hr at 37°C with shaking (Room temp [RT] is also sufficient).
6. Proceed to immunostaining or store at 4°C.

Antibody incubation (use a microwell dish in order to conserve reagents)

7. Using a mouth pipette, carefully remove as much of the washing buffer as possible, leaving just the follicles in each well.
8. Add 8-10 µL of primary antibody to the sample and incubate at 37°C for 1 hour (RT 3-4 hours or 4°C overnight). Antibodies are diluted in wash buffer; 1:100 dilution is commonly recommended as a starting dilution for most antibodies.
9. Wash follicles in wash buffer at least three times, 10 min each, by transferring to a new well with fresh wash buffer.
10. Add 8-10 µL secondary antibody to the sample and incubate at 37°C for 1 hour. (1:500 – 1:1000 dilution for most Alexa Fluor conjugated secondaries.) Phalloidin conjugates can be added directly to secondary antibody solution for simultaneous staining (1:50 works well). Be sure to protect samples from light from this point on.
11. Wash follicles in wash buffer at least three times, 10 min each.
12. (Optional) If using a nuclear stain that is not included in the mounting medium, incubate the follicles at 37°C for 10-30 min depending on the dye (see manufacturer's recommendations). Wash follicles in wash buffer at least three times, 10 min each.

Mounting the follicles (I prefer to do this on a dissecting scope)

13. Prepare a clean slide by placing 4 dots of valap or Vaseline where the corners of the coverslip will be. This holds the coverslip off of the slide in order to prevent the follicles from being crushed.
14. Transfer follicles to the center of the square created by valap dots. Remove excess wash buffer with mouth pipette. Add a small drop (about 10-20 µL) of mounting media to the sample.
15. Carefully place the coverslip on the valap dots and while watching through the scope, push down on alternating corners until the follicles stop shifting around. Do this carefully and slowly as you risk crushing the follicles. You want them pinned down so they don't move but not crushed.
16. Add more mounting media by pipeting at the side of the coverslip until the entire area of the coverslip is filled. If the follicles shift at this point, you can press the coverslip down a bit more but it probably isn't necessary.
17. Seal the coverslip to the slide using clear nail polish.
18. Slides can be stored at 4°C. Warm to RT before imaging.