Protocol for IF staining for γ-H2AX and 53BP1 DNA Damage Response Proteins

Reagents and antibodies for staining:

- 0.1% Triton X-100 (40 μL Triton into 40 mL of PBS)
- PBS-T (100 uL Tween-20 into 100 mL PBS)
- Blocking solution (1 mL normal donkey serum [*Sigma D9663-10ML]* into 9 mL PBS-T)
- DAPI (1 ug/mL in H_2O) *Life technologies D1306*
- Glycergel mounting medium *Dako C0563*

Antibody	Manufacturer	Dilution
Mouse Anti-gamma H2AX	*EMD Millipore 05-636*	1:500
Rabbit Anti-53BP1	*Novus Biologicals NB100-304*	1:500
Goat Anti-Salmonella	*SeraCare 5310-0323*	1:500
Donkey anti-mouse Alexa 647	*ThermoFisher A-31571*	1:200
Donkey anti-rabbit Alexa 555	*ThermoFisher A-31572*	1:500
Donkey anti-goat Alexa 488	*ThermoFisher A-11055*	1:200

Preparing slides for IF:

1. Fix slides with 4% paraformaldehyde (remove spent media with the Pasteur Pipette and vacuum pump).
    a. *Tip: Formaldehyde is also acceptable. It is important that this solution be prepared fresh (prepared the same day is best, but within 1 week is okay)*
    b. *PFA and formaldehyde are toxic chemicals, use caution when handling these.*
2. Wash 1X with pre-warmed PBS before adding 1 mL of 4% PFA and incubate at RT for 15-30 minutes).
    a. *Note: I have tested fixation at room temperature and at 37°C. I recommend fixation at room temperature and not at 37°C. Fixation at 37°C leads to sub-optimal results if your slides are going to be stored in PBS for several weeks.*
3. Remove paraformaldehyde with a stripette and add to hazardous waste receptacle.
4. Replace with 1 mL of PBS. Once in PBS, slides can be stored @ 4C for up to 2 months.
    a. *Note: I have tested and confirmed there isn't really a decrease in foci staining if you store your slides at 4°C for >2 weeks*
5. Carefully remove slides from plate (using a scalpel blade and tweezers) and place on an empty P1000 pipette tip box. Immediately add 100 μL of PBS to prevent the cover slip from drying out.
    a. *Note: Make sure that the slide is kept with the cell-side up. Be careful to avoid having the coverslip completely dry.*
6. Remove PBS by holding the coverslip with tweezers and gently touching the tip of the coverslip to a kim wipe to 'wick' away the PBS.
7. Wash coverslips 2 more additional times with 100 μL PBS.
8. Permeabilize cells with 100 μL of 0.1% Triton X-100 in PBS at room temperature for 10 mins.
9. Remove the 0.1% Triton X-100 solution using a kim wipe, and replace with 100 μL of blocking solution (10% normal goat serum in 0.1% PBST). Incubate at room temperature for 1 hour.
10. Remove blocking solution and add your primary antibodies diluted in blocking solution and incubate at room temperature for 1 hour.
11. Aspirate and wash slides with 0.1% PBS-T three times.
12. Incubate with secondary antibodies for 1 hr at room temperature.
13. Aspirate and wash slides with 0.1% PBS-T three times.
14. Remove PBS-T and counterstain with DAPI (1 μg/mL): incubate for 1-5 mins at room temperature .
    a. DAPI is a known mutagen. Always be careful when handling this solution.
15. Remove DAPI and add 100 μL of water.
    a. *Note: DAPI is insoluble in PBS. DO NOT add PBS to the slides, but instead use H_2O,*
16. Glue slides to microscope slides with Dako Glycergel and let dry (O/N works best). Store in dark and image using Zeiss 710 confocal.
    a. *Tip: You can pre-heat the glycergel in the microwave for 8-10 seconds.*

Troubleshooting

I) No signal in positive control: Make sure that the settings (i.e. gain, laser intensity, etc.) are correct. Sometimes the software will unexpectedly change your saved settings.

II) Noticeable background signal: Increase the number of washes with PBS-T, or alternatively you can increase the amount of time that you wash for (i.e. incubate with PBS-T for 5 minutes per wash).