Protocol

Iterative Indirect Immunofluorescence Imaging (4i)

Accompanying the publication and adapted from the publication:

G. Gut et al., Science 361, eaar7042 (2018). DOI: 10.1126/science.aar7042

for 384-well plates

Materials

- Phosphate-buffered saline, PBS, home-made
- Paraformaldehyde, PFA, Electron Microscopy Sciences
- Bovine Serum Albumin, BSA, Sigma Aldrich
- Amonium chloride, NH_4Cl, Sigma Aldrich
- Maleimide, Sigma Aldrich
- N-Acetyl-Cysteine, NAC, Sigma Aldrich
- Urea, Sigma Aldrich
- Guanidinum chloride, GC, Sigma Aldrich,
- Tris(2-carboxyethyl)phosphine hydrochloride, TCEP, Sigma Aldrich
- L-Glycine, Sigma Aldrich
- 4', 6-Diamidino-2-phenylindole, DAPI, Lifetechnologies

Buffers

Imaging buffer: **IB** used to prevent photocrosslinking during imaging.

700mM NAC in ddH_2O.
Adjust to pH7.4. Currently buffer system in place.
1x solution

Elution buffer: **EB** used to elute antibodies from sample.

0.5M L-Glycine,
3M Urea,
3M GC,
70mM TCEP, **add TCEP just before Elution**
All in ddH_20. Adjust to pH2.5.
1x solution

Blocking solutions: **Conventional Blocking Solution (cBS)** used for antibody staining.

2% BSA,
200mM NH_4Cl
All in PBS.
2x solution

4i Blocking Solution (sBS) used for sample blocking.

2% BSA,
200mM NH_4Cl,
300mM Maleimide,
All in PBS.
sBS is made freshly just before the blocking step.
2x solution

DNA stain solution: **DSS** used amongst other for image registration.

100μg/ml DAPI, 1/125 - 1/25
All in PBS.
2x solution

Antibody solutions: **Primary antibody solution (PAS)** used to hybridize prim. ab.

Unlabeled, primary antibody (2 species), 1/100-1/1000
All in cBS.
2x solutions

Secondary antibody solution (SAS) used to hybridize sec. ab.

Labeled, secondary antibody (2 species), 1/150-1/500
All in cBS.
2x solutions

Protocol

CAUTION: Protect the sample from light whilst secondary antibodies are hybridized and Imaging Buffer is not added.

Protocol consists of 4 parts: sample preparation, antibody elution, immunofluorescence, and imaging.

All steps are performed in a 384-well plate. If you choose other cell carriers (e.g. glass slides), you may want to adjust the concentrations of the 2x solutions to 1x.

Definitions:
Wash with X, where X can be H_2O or PBS.

The following sequence is repeated 6 times:
a. Aspiration to 15μl per well.
b. Dispensing of 95μl of solution per well.

Typical workflow

1. Sample preparation
2. Antibody elution
3. Immunofluorescence
4. Imaging
5. Repeat step 2 and 4 ad libitum.
6. Enjoy multiplexed images!

Sample preparation

1. Fixation
   Cells are fixed with 4%PFA in PBS (freshly prepared on the day) for 30 min.

2. Wash with PBS. Aspiration to 30 μl residual volume per well.

3. Permeabilization
   Dispense 30μl per well 1% TritonX-100 in PBS, incubate 15 min.

4. Wash with PBS. Aspiration to 30μl residual volume per well.

Antibody elution

1. Wash with H_2O. Aspiration to 15µl residual volume per well.

2. Elution
The following sequence is repeated 3 times:
a. Dispense 50µl of EB per well, incubate 10 min, shaking 150rpm.
b. Aspirate to 15µl residual volume per well.

3. Wash with PBS. Aspiration to 30µl residual volume per well.

Immunofluorescence

1. Blocking
Dispense 30µl per well sBS, incubate 1h, shaking 100RPM.

2. Wash with PBS. Aspiration to 30µl residual volume per well.

3. Primary antibody
Dispense 30µl of PAS per well incubate 2h, shaking 100RPM.

4. Wash with PBS. Aspiration to 30µl residual volume per well.

5. Secondary antibody
Dispense 30µl of SAS per well, incubate 1h, shaking 100RPM.

6. Wash with PBS. Aspiration to 30µl residual volume per well.

7. Nuclear staining
Dispense 30µl of DSS per well, incubate 10 min, shaking 100RPM.

8. Wash with H_2O. Aspiration to 15µl residual volume per well.

9. Preparation for imaging
Dispense 100µl of IB per well. Image.

Imaging

Image your sample as you are used to. Avoiding long exposures at high laser/lamp power as this may result in difficult antibody elution.