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**CycIF (chemical fluorophore inactivation) protocol, v0.2**

The protocol below has been optimized for adherent cells grown in 96-well plates.

Cell lines tested to date: MCF7, MCF10A, Colo858, RPE.

**Reagents:**

* 4% paraformaldehyde/PFA (diluted from the 16% stock from Electron Microscopy Services, cat. #15710)
* ice-cold methanol
* Hoechst 33342 (Invitrogen, cat. #H3570)
* Odyssey blocking buffer (LI-COR Biosciences, cat. #927-40001)
* 1x PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4 and 1.8 mM KH2PO4)
* 30% hydrogen peroxide (Sigma-Aldrich, cat. #H1009)
* 1 M NaOH (dissolve from pellets; Sigma-Aldrich, cat. #S5881)
* 2 M HCl (dilute from 12 M solution; Sigma-Aldrich, cat. #258148)

**Fluor-inactivation solution for Alexa dyes**:
3% H2O2 (1/10 dilution from 30% stock), 20 mM NaOH (1/50 dilution from 1 M stock) in PBS
*For indirect IF (1o+2 o Abs), use up to 4.5%* H2O2 *and 25 mM NaOH if necessary.*

**Fluor-inactivation solution for GFP/YFP/mCherry fusion proteins**:
3% H2O2 (1/10 dilution from 30% stock), 20 mM HCl (1/100 dilution from 2M stock) in PBS
*For Turquoise/ECFP fusion proteins, increase inactivation time to 2 hours (or bleach twice).*

**Procedure:**

1. Fixation: Fix cells in 96-well plates with 140 ul 4% PFA at room temperature for 30 minutes. Then wash 4 times with 250 ul PBS per wash.
*\*Extensive fixation is required. For loosely-adherent cells the fixation time may be as long as 1 hr.*
2. Permeabilization: Add 140 ul ice-cold methanol, and allow the plates to sit at room temperature for 10 minutes. Then wash 4 times with 250 ul PBS per wash.
3. Blocking: Incubate the cells with 50 ul Odyssey blocking buffer at room temperature for 1 hr.
4. Staining: Dilute antibodies in 50 ul Odyssey buffer, and add to the cells. Incubated at 4oC overnight, and then wash 4 times with 250 ul PBS per wash.
\**All washes should be as gentle as possible. A plate washer (e.g. Bio-Tek EL406) is recommended.*
5. Hoechst staining: Dilute Hoechst 33342 (1mg/ml stock) 1:5000 in 140 ul PBS and incubate with the cells for 15 minutes at room temperature. Then wash 4 times with 250 ul PBS per wash.
6. Imaging: Use a Cytell (GE Healthcare Life Sciences) or Operetta (Perkin Elmer) imager or the equivalent. For Cytell, the fixed filter setting is required for 4-channel immunofluorescence (Hoechst, 488 nm, 555 nm, and 647 nm) to minimize crosstalk. In addition, a binning option or high-sensitivity camera (e.g. EMCCD, sCMOS) may be required for weak signals, such as from directly-conjugated antibodies.
7. Fluorophore inactivation: After imaging, remove the PBS and add 140 ul fluor-inactivation solution. Incubate at room temperature for 1 hr with light (using a tabletop lamp or equivalent). Then wash 4 times with 250 ul PBS per wash. (After washing, the cells can be stored at 4oC before proceeding, but it is best to proceed with the next round of imaging as soon as possible.)
*\* Light treatment is optional, but it may increase the efficiency of fluorophore inactivation ~10-20%, depending on the light source.*
8. Start CycIF: After fluor inactivation, you may begin a second round of labeling starting from step 3 (blocking) or step 4 (staining). However, imaging after inactivation and before the next round of labeling is recommended to ensure complete removal of dye signal.

**Notes:**

1. A set of primary/secondary antibodies can be added during the first labeling cycle. However, prolonged fluor inactivation (~1.5 hours) may be required to completely remove the amplified fluorescent signals.
2. Image registration (alignment) will be required for merging signals from different rounds of CycIF, and you must ensure that your imager has a robust stage/position control. Only a portion of each image is retained during image registration, so stitching of images from overlapping sites/fields before registration will allow a larger number of individual cells to be retained in the final registered image for downstream image analysis.
3. If possible, all wash steps should be performed on an automatic plate washer at the lowest setting. However, hand washing with care also can work.
4. For any technical questions or suggestions/feedback, please contact Jerry Lin (Sorger Lab/Laboratory of System Pharmacology, Harvard Medical School) at jia-ren\_lin@hms.havard.edu. Additional information about CycIF also can be found on the HMS LINCS website at <http://lincs.hms.harvard.edu/lin-NatCommun-2015/>, including an up-to-date list of antibodies and dyes tested in the Sorger lab.