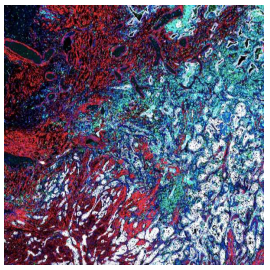
Jun 12, 2024

Tissue Cyclic Immunofluorescence (t-CyCIF)_PCA2024_spacer



Forked from Tissue Cyclic Immunofluorescence (t-CyCIF)_PCA2020

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Protocol status: Working
We use this protocol and it's working

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Abstract

The architecture of normal and diseased tissues strongly influences the development and progression of disease as well as responsiveness and resistance to therapy. We describe a tissue-based cyclic immunofluorescence (t-CyCIF) method for highly multiplexed immunofluorescence imaging of specimens mounted on glass slides. t-CyCIF generates up to 60-plex images using an iterative process (a cycle) in which conventional low-plex fluorescence images are repeatedly collected from the same sample and then assembled into a high dimensional representation. t-CyCIF requires no specialized instruments or reagents and is compatible with super-resolution imaging; we demonstrate its application to quantifying signal transduction cascades, tumor antigens and immune markers in diverse tissues and tumors. The simplicity and adaptability of t-CyCIF makes it an effective method for pre-clinical and clinical research and a natural complement to single-cell genomics.

Key resources, reagents, and software are listed at the HMS LINCS Center Publication Page http://lincs.hms.harvard.edu/lin-elife-2018/ (RRID:SCR_016370). This page provides links to an OMERO image database from which individual images can be obtained; stitched and registered image panels can be obtained at **www.cycif.org** (RRID:SCR_016267) and a video illustrating the t-CyCIF method can be found at https://vimeo.com/269885646.

This protocol is used in the following manuscripts:

- Lin J-R, Izar B, Wang S, Yapp C, Mei S, Shah P, Santagata S, Sorger PK. (2018). Highly multiplexed immunofluorescence imaging of human tissues and tumors using t-CyCIF and conventional optical microscopes. eLife. PMID: 29993362
- Du Z, Lin JR, Rashid R, Maliga Z, Wang S, Aster J, Izar B, Sorger PK, Santagata S. (2019). Qualifying antibodies for image-based immune profiling and multiplexed tissue imaging. Nature Protocols. PMID: 31534232

The original t-CyCIF protocol can be found at **dx.doi.org/10.17504/protocols.io.rpxd5pn**.

Materials

MATERIALS

- Hydrogen peroxide solution contains inhibitor, 30 wt. % in H2O, ACS reagent **Sigma Aldrich Catalog #**216763-500ML
- 1N NaOH
- 20X Phosphate Buffered Saline **Thermo Fisher Scientific Catalog #**28348
- UltraPure Glycerol **Life Technologies Catalog #**15514011
- Hoechst 33342 **Cell Signaling Technology Catalog #**4082
- Platinum Coverslips (24x50)/Cs **American Master Tech Scientific Catalog #**GL2450
- StainTray 10 Slide Tray (Black) **American Master Tech Scientific Catalog #**LWS10BK
- Tissue-Tek® Vertical 24 Slide Rack **American Master Tech Scientific Catalog #**LWS2124
- Tissue-Tek Slide Staining Set (Dishes and Baths) **American Master Tech Scientific Catalog #**LWS19
- Portable 20000 LUX Dimmable LED Bright Light Panel **Amazon**
- Graduated Glass Cylinder 100 mL **American Master Tech Scientific Catalog #**LWG0726
- Centrifuge Tubes 15 mL **Corning Catalog #**430790
- Centrifuge tubes 50 mL **Catalog #**430808
- SuperBlock™ (PBS) Blocking Buffer **Thermo Fisher Catalog #**37515
- iSpacer 0.15mm **SUNjin Lab Catalog ##**IS111:250pieces

Additional Materials:

- Antibodies (experiment-specific)
- Deionized water
- Ice box
- Pipettes
- Pipette tips

Protocol materials

UltraPure Glycerol **Life Technologies Catalog #**15514011 Materials

Graduated Glass Cylinder 100 mL **American Master Tech Scientific Catalog #**LWG0726 Materials

20X Phosphate Buffered Saline **Thermo Fisher Scientific Catalog #**28348 Materials

Centrifuge tubes 50 mL **Catalog #**430808 Materials

1N NaOH Materials

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SuperBlock™ (PBS) Blocking Buffer **Thermo Fisher Catalog #**37515 Materials, Step 9

StainTray 10 Slide Tray (Black) **American Master Tech Scientific Catalog #**LWS10BK Materials

Tissue-Tek® Vertical 24 Slide Rack **American Master Tech Scientific Catalog #**LWS2124 Materials

Tissue-Tek Slide Staining Set (Dishes and Baths) **American Master Tech Scientific Catalog #**LWS19 Materials

iSpacer 0.15mm **SUNjin Lab Optical Clearing Innovation Co. Catalog ##**IS111:250pieces Materials, Step 5.2

Hydrogen peroxide solution contains inhibitor, 30 wt. % in H2O, ACS reagent **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**216763-500ML

Materials

Before start

Note that t-CyCIF is optimized for FFPE specimens, which must first undergo dewaxing and antigen retrieval to expose antigenic sites for antibody binding. An example of an automated version of this process can be found here: **dx.doi.org/10.17504/protocols.io.4zpgx5n**.

Pre-Staining and Background Determination

1 Pre-staining and Background Determination takes approximately 16-24 hours.

Make fluorophore bleaching solution. Combine 165 mL 1X PBS, 30 mL 30% (wt/vol) H_2O_2, and 4 mL [M] 1 Molarity (M) NaOH. The final working concentration is 4.5% (wt/vol) H_2O_2 and [M] 20 millimolar (mM) NaOH in PBS. 199 mL of bleaching solution is enough for 30 standard slides.

- **CRITICAL STEP** Fluorophore bleaching solution should be prepared immediately before use.

2 Place slides flat in a plastic transparent container with the tissue facing up, and then gently pour fluorophore bleaching solution into the container to completely cover tissue. Sandwich the container between two LED light panels (one above and one below) at Room temperature for 2x 00:30:00 . 30m

- **CRITICAL STEP** The pre-bleaching step is critical for reducing autofluorescence in the tissue and to inactivate the fluorophores of the secondary antibody from the pre-staining step.
- **CRITICAL STEP** Light sources that produce excessive heat can damage tissues. LED light sources are therefore preferable, and large flat LED panels are now readily available at low cost (see "**Materials**" for our preferred light panel).
- **CRITICAL STEP** Completely immerse the tissue sections in fluorophore bleaching solution. During the subsequent bleaching process, bubbles will appear and gradually increase in size and number. This indicates that the oxidation reaction is proceeding as expected.

3 Wash slides 4 times with 1X PBS at Room temperature for at least 00:05:00 per wash. Slides can be placed into a slide rack and lowered into a staining dish of PBS. 5m

4 Place slides in the slide tray, completely cover tissues with the secondary antibody solution used in the pre-staining procedure with added Hoechst solution (2.5 µg/ml) and incubate in the dark at 4 °C overnight to block tissues from non-specific binding.

- **CRITICAL STEP** Place damp paper towels in the slide tray to maintain humidity and prevent evaporation of the antibody solution.
- **CRITICAL STEP** Do not use a hydrophobic barrier pen on the slides, as we have found that this adversely affects results.
- **CRITICAL STEP** Be careful not to scratch the tissue with pipette tip when applying the antibody solution.

5 Prepare slides for cyclic imaging.

5.1 Wash slides 4 times with 1X PBS at Room temperature for at least 00:05:00 per wash. 5m

5.2 Using a lint-free wipe, remove excess PBS from around the tissue. Carefully apply self-adhesive iSpacer 0.15mm **SUNjin Lab Optical Clearing Innovation Co. Catalog ##**IS111:250pieces onto the slide surrounding the tissue. If not imaging background, place slides in PBS while making the fresh bleach solution for used in step 7.

6 **OPTIONAL** Image background/autofluorescence

6.1 Mount coverslips onto slides with 200 µL of 50% (vol:vol) glycerol in 1X PBS to prevent dehydration during imaging. Carefully position coverslips over the center of each slide and lower slowly onto the slide to avoid producing bubbles between the coverslip and to prevent scratching tissues. Do not allow coverslip to overhang the edge of the slide. Dry excess liquid by gently pressing the long edges of the slide against a paper towel.

- **CRITICAL STEP** Wet-mounting and positioning coverslips takes some practice that should be undertaken initially using non-precious specimens.

6.2 Image background staining/autofluorescence using ex/em filters that will be used in the rest of the experiment.

6.3 Place slides in 1x PBS pre-warmed to 42°C until coverslips fall off the tissue - up to 1h. Do not disturb the tissue spacer - it will remain on the slide through the remaining steps of the experiment.

7 Bleach slides at least 2x 00:30:00 at Room temperature as previously described.

8 Wash the slides 4 times with 1X PBS at Room temperature for 00:05:00 per wash. 5m

- **CRITICAL STEP** ensure PBS covers entire slide including label region to ensure all bleaching solution is removed from slides.

- **PAUSE POINT** Slides may be stored in 1X PBS at 4 °C for several days. Avoid letting tissue dry; ensure entire tissue is covered in 1X PBS.

First (AND/OR second) Round of t-CyCIF (Indirect IF)

9 The first round of t-CyCIF takes approximately 16-24 hours.

Dilute up to three unconjugated primary antibodies from different species to the appropriate concentration in SuperBlock™ (PBS) Blocking Buffer **Thermo Fisher Catalog #**37515 containing Hoechst 33342 (2.5 µg/ml), cover tissue, and incubate in the dark at 4 °C overnight.

- **CRITICAL STEP** Unconjugated antibodies used in CyCIF are limited by unique secondary antibody and flourophore combinations available; we limit indirect IF to first two cycles).The optimal dilution for primary antibodies must be optimized empirically; we usually test across a range of dilutions starting from 1:100, guided by manufacturer's recommendations. The times listed for antibody incubation can be adjusted empirically; we use long incubations at 4 °C for convenience. (See Lin et al., 2018 and https://www.cycif.org/ (RRID:SCR_016267) for information on increasing the throughput of t-CyCIF experiments.)

10 Wash slides 2 times with 1X PBS at Room temperature for 00:10:00 (max 5 min) per wash. The longer slides are washed/rinsed minimizes unbound antibody artifacts. 10m

11 Cover the tissue with secondary antibodies diluted in SuperBlock Blocking Buffer containing Hoechst (2.5 µg/ml) . Incubate in the dark at Room temperature for 01:00:00 . 1h

12 Wash slides 3 times with 1X PBS at Room temperature for 00:30:00 per wash. 30m

13 Mount coverslips on slides with 200 µL of 50% glycerol in PBS and image as described.

- **CRITICAL STEP** Use a saved ROI in the imaging software so that the exact same region of tissue is imaged for every cycle of t-CyCIF.
- **TROUBLESHOOTING** Weak signal. Possible reason: Low signal can result because of low level antigen expression. Direct immunofluorescence using conjugated antibodies does not provide the signal amplification that can be generated in indirect immunofluorescence. Solution: Increase the exposure time while acquiring image; increase the antibody concentration during staining step; use the corresponding unconjugated antibodies in the

first round instead of the conjugated antibody to see if signal amplification from indirect immunofluorescence improves signal; if necessary, find an alternative antibody.

- **TROUBLESHOOTING** Saturating signal. Possible reason: Abundant antigen in sample or excessive amount of antibody. Solution: Decrease the antibody concentration used during the staining steps; decrease the incubation time of the sample with antibody; decrease the exposure time during image acquisition.

14 After imaging, remove the coverslips as described above and wash the slides 2 times with 1X PBS at Room temperature for 00:03:00 (max 5 min) per wash.

15 Perform fluorophore bleaching for 2x 00:30:00 at Room temperature as described. 30m

16 Wash slides 4 times with 1X PBS at Room temperature for 00:05:00 per wash. 5m

- **CRITICAL STEP** Wash slides thoroughly to remove fluorophore bleaching solution, since carry-over can adversely affect subsequent t-CyCIF cycles.
- **PAUSE POINT** Slides may be stored in 1X PBS at 4 °C for several days. Make sure the entire tissue is covered in 1X PBS. Otherwise, the tissue may become dry and yield poor staining results.

Subsequent Cycles of t-CyCIF (Direct IF) 1h 3m

17 Subsequent cycles of t-CyCIF use antibodies with directly conjugated fluorophores and each takes approximately 16-24 hours each. The maximum number of cycles for t-CyCIF is determined by how long the tissue structure is preserved between cycles. We are able to perform >10 cycles for most tissue types.

Dilute up to three conjugated antibodies conjugated with different fluorophores in SuperBlock® (PBS) Blocking Buffer containing Hoechst (2.5 µg/ml). Cover tissue with antibody solution and incubate in the dark moisturized container at 4 °C overnight.

- **CRITICAL STEP** We use Alexa Fluor 488-, Alexa Fluor 555-, and Alexa Fluor 647- conjugated primary antibodies. Dilution is optimized empirically starting from 1:100 (vol:vol).
- **CRITICAL STEP** Alexa Fluor 546-, Alexa Fluor 568-, and Alexa Fluor 594- conjugated secondary antibodies are difficult to bleach; Avoid use.

18 Wash the slides 3 times with 1X PBS at Room temperature for 00:30:00 per wash. 30m

19 Mount coverslips with 200 µL of 70% (vol:vol) glycerol in PBS and image the saved ROI with the RareCyte CyteFinder as described.

20 Remove the coverslips as described.

21 Wash the slides 2 times with 1X PBS at Room temperature for 00:03:00 per wash. 3m

22 Bleach slides for 2x 00:30:00 at Room temperature as described. 30m

23 Wash the slides 4 times with 1X PBS at Room temperature for 00:03:00 per wash.

24 Start next t-CyCIF cycle: repeat steps 16-22 for each additional cycle.

- **TROUBLESHOOTING** Blurry images.
- **TROUBLESHOOTING** Weak signal.
- **TROUBLESHOOTING** Saturating signal.
- **TROUBLESHOOTING** Cell loss. Possible reason: Difficult tissue type (very low cell density). Insufficient tissue fixation. Damage from t-CyCIF procedure (*e.g.* rough handling of samples during washing). Solution: Check pre-analytical variables. Cautious handling of samples during application of antibodies and washing steps as well as during manipulation of coverslips.
- **TROUBLESHOOTING** Signal present after fluorophore bleaching step. Possible reason: Insufficient fluorophore inactivation. Solution: Avoid Alexa Fluor 546-, Alexa Fluor 568-, and Alexa Fluor 594- conjugated antibodies because they are difficult to inactivate; dilute conjugated antibodies further; extend fluorophore bleaching time; check that light is hitting the sample.