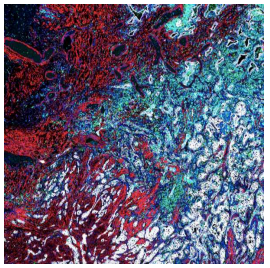
Oct 17, 2022 Version 2

Tissue Cyclic Immunofluorescence (t-CyCIF) version 3 V.2



Version 1 is forked from Tissue Cyclic Immunofluorescence (t-CyCIF)

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Cyclic Immunofluoresce... NCIHTAN

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Manuscript citation:
Muhlich JL, Chen Y, Yapp C, Russell D, Santagata S, Sorger PK, Stitching and registering highly multiplexed whole-slide images of tissues and tumors using ASHLAR. Bioinformatics 38(19). doi: 10.1093/bioinformatics/btac544

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 50 wt. % in H2O, stabilized **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**516813-500ML
- 1N NaOH **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**1091371000
- DPBS (10X), no calcium, no magnesium **Thermo Fisher Scientific Catalog #**14200083
- UltraPure Glycerol **Life Technologies Catalog #**15514011
- Hoechst 33342 **Cell Signaling Technology Catalog #**4082
- Coverslips thickness 1 1/2 24×50 mm **Corning Catalog #**2980-245
- 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
- Light Therapy Lamp - UV Free Bright White Led Therapy Light with 15000 LUX **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

Additional Materials:

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

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 25 mL 1X PBS, 4.5 mL 30% (wt/vol) H_2O_2, and 0.8 mL [M] 1 Molarity (M) NaOH in a 50-ml centrifuge tube. The final working concentration is 4.5% (wt/vol) H_2O_2 and [M] 20 millimolar (mM) NaOH in PBS. 30 mL fluorophore bleaching solution is enough for four 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. Place the container between two **LED light panels** (one above and one below) at Room temperature for 00:45:00 .

- **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 00:03:00 (max 5 min) per wash. Slides can be placed into a slide rack and lowered into a staining dish of PBS.

4 Place slides in the slide tray, cover all tissues with the secondary antibody solution used in the pre-staining procedure to and incubate in the dark at 4 °C overnight to block 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 subsequent cycles.
- **CRITICAL STEP** Be careful not to scratch the tissue with pipette tip when applying the antibody solution.

5 Bleach the fluorophores for 00:30:00 at Room temperature as described.

6 Wash the slides 4 times with 1X PBS at Room temperature for 00:03:00 (max 5 min) per wash.

- **CRITICAL STEP** Wash the slides to remove the fluorophore bleaching solution completely which may affect subsequent t-CyCIF steps.

7 Incubate slides with Hoechst solution (2.5 µg/ml) in the dark at Room temperature for 00:10:00 .

8 Wash the slides 4 times with 1X PBS at Room temperature for 00:03:00 (max 5 min) per wash.

9 Mount coverslips onto slides with 150 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.

10 Load the slide into the RareCyte CyteFinder and image at each wavelength to record the background signal.

- **CRITICAL STEP** Typically only a portion of each slide is covered in tissue and only this region should be scanned; it is important to save this region of interest (ROI) in the imaging software so that precisely the same region can be imaged in subsequent rounds of t-CyCIF.
- **CRITICAL STEP** Check images as they are being acquired and adjust exposure times to remain in a linear range.
- **TROUBLESHOOTING** Blurry images. Possible reason: The slides are not flat and focusing is suboptimal. Solution: Examine the slide holder for precipitate and remove; ensure that slides are loaded properly in the slide holder; change coverslips; adjust the focusing points.

11 After image acquisition, remove coverslips by placing the slides in 1X PBS in a staining dish (which holds slides vertically) for 00:10:00 and then slowly pull the slides vertically out of the solution allowing the coverslip to remain behind.

- **CRITICAL STEP** De-coverslipping is another procedure that requires practice. Always allow coverslips to fall away through gravity. Do not push the coverslips as this will scratch and damage tissues. It may take longer time than the recommended 00:10:00 for coverslips to detach.

12 Wash the slides 4 times with 1X PBS at Room temperature for 00:03:00 (max 5 min) per wash.

■ **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 will dry out resulting in poor results in subsequent staining steps.

First Round of t-CyCIF

13 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 Blocking Buffer** with 1ug/ml Hoechst 33342, cover all the tissue, and incubate in the dark at 4 °C overnight.

- **CRITICAL STEP** In the first round of t-CyCIF, unconjugated primary antibodies can be used. As in conventional immuno-fluorescence, these antibodies must be from

different species (e.g. rabbit, mouse, and rat) to allow for detection with species-specific secondary antibodies. 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.)

14 Wash slides 4 times with 1X PBS at Room temperature for 00:03:00 (max 5 min) per wash.

15 Cover the tissue with secondary antibodies and incubate in the dark at Room temperature for 02:00:00 .

16 Wash slides 4 times with 1X PBS at Room temperature for 00:03:00 (max 5 min) per wash.

17 Mount coverslips onto slides with 150 µL of 50% glycerol in PBS and image the saved ROI for each slide with the RareCyte CyteFinder as described.

- **CRITICAL STEP** Use the saved ROI in the imaging software so that the exact same region of tissue is imaged for every cycle of t-CyCIF.
- **TROUBLESHOOTING** Blurry images (See above).
- **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.

18 After imaging, remove the coverslips as described and wash the slides 4 times with 1X PBS at Room temperature for 00:03:00 (max 5 min) per wash.

19 Perform fluorophore bleaching for 00:45:00 at Room temperature as described.

20 Wash slides 4 times with 1X PBS at Room temperature for 00:03:00 (max 5 min) per wash.

- **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

21 Subsequent cycles of t-CyCIF take approximately 16-24 hours each. The maximum number of cycles for t-CyCIF depends on tissue type, which is evaluated by counting nuclei in the Hoechst channel. We are able to perform >10 cycles for most tissue types and >20 cycles for some resilient tissues, such as tonsil.

Dilute up to three conjugated antibodies conjugated with different fluorophores in **SuperBlock (PBS) Blocking Buffer** with 1ug/ml Hoechst 33342. Cover all tissue with antibody solution and incubate in the dark at 4 °C overnight.

- **CRITICAL STEP** We typically 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** Avoid using Alexa Fluor 546-, Alexa Fluor 568-, and Alexa Fluor 594-conjugated secondary antibodies, as these fluorophores are difficult to bleach.

22 Wash the slides 4 times with 1X PBS at Room temperature for 00:03:00 (max 5 min) per wash.

23 Wash the slides 4 times with 1X PBS at Room temperature for 00:03:00 (max 5 min) per wash.

Subsequent Cycles of t-CyCIF

24 Mount coverslips onto slides with 150 µL of 50% (vol:vol) glycerol in PBS and image the saved ROI with the RareCyte CyteFinder as described.

25 Remove the coverslips as described.

26 Wash the slides 4 times with 1X PBS at Room temperature for 03:00:00 (max 5 min) per wash.

27 Bleach the fluorophores for 00:45:00 at Room temperature as described.

28 Wash the slides 4 times with 1X PBS at Room temperature for 00:03:00 (max 5 min) per wash.

29 Start next t-CyCIF cycle: repeat steps 22 on 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.