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 We use this protocol and it's working

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🌐 Antibody Conjugation and CODEX Multiplexed Immunofluorescence of Fresh Frozen Tissue

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ABSTRACT

This protocol describes antibody conjugation, tissue preparation, and microscopy for CODEX multiplexed immunofluorescence analysis of fresh frozen tissue.

GUIDELINES

Wear PPE and work in a well ventilated area.

MATERIALS

10X CODEX Buffer, Akoya Biosciences, 7000001
 CODEX Assay Reagent, Akoya Biosciences, 7000002
 CODEX Conjugation Kit, Akoya Biosciences, 7000009
 CODEX Staining Kit, Akoya Biosciences, 7000008
 CODEX Gaskets v2, Akoya Biosciences, 7000010
 Purified antibodies
 Fresh frozen tissue on 0.1% Poly-L-Lysine coated coverslips (22mm X 22 mm x 1.5mm)
 BSA Removal Kit, Abcam, ab173231- only needed for BSA removal from antibodies
 50kDa MWCO filter, EMD Millipore, UFC505096
 Molecular Probes DAPI (4',6 Diamidino 2 Phenylindole, Dihydrochloride), Thermo Fisher Scientific, D1306
 Dumont Cover Slip Forceps - Dumoxel, Fine Science Tools, 11251-33
 6-well TC plates-do not need to be tissue culture treated, VWR, 10861-554
 96 well plates, Akoya Biosciences, 7000006
 4% formaldehyde, methanol-free, Thermo Fisher Scientific, R37814
 Methanol free formaldehyde ampules, Thermo Fisher Scientific, 28908
 1X PBS, Life Technologies, 14190144
 Ambion Nuclease-Free Water, Thermo Fisher Scientific, 43-879-36
 Methanol, Sigma-Aldrich, 34860-1L-R
 DMSO - ACS reagent, ≥99.9%, Sigma-Aldrich, 472301-4L
 Acetone, HPLC Plus, for HPLC, GC, and residue analysis, ≥99.9%, Sigma-Aldrich, 650501-4X4L
 Microcentrifuge

SAFETY WARNINGS

DMSO readily enters the skin and can carry dissolved chemicals with it.

Organic solvents and paraformaldehyde are aspiration hazards, and it is recommended that work with them be carried out in a fume hood. Organic solvents also pose flammability hazards.

Chemical handling, storage, and disposal information can be found in Safety Data Sheets (SDS).

Antibody Conjugation for Custom Antibody Markers

1 Start with purified antibodies (no BSA, azides, glycerol, etc.). If BSA is present, remove with ab173231.

2 Block 50 kDa molecular weight cut off filters with 500 µL of filter blocking solution.

3 Centrifuge at 12000 x g for 00:02:00 .

4 Dilute 50 µg of each antibody into 100 µL of PBS and filter with 50 MW cut off filters.

5 Centrifuge at 12000 x g for 00:08:00 .

6 Reduce antibodies with the reduction mixture for 20-25 minutes at ~ 20 °C .

Note

25 minutes of reduction is suitable for many antibodies but may need to be optimized (15 min-40 min) depending on the primary antibody used. In general, shorter reduction times are more appropriate than longer times.

7 Centrifuge the reduced antibodies at 12000 x g for 00:08:00 and add buffer solution.

8 Rehydrate oligonucleotide barcodes in 10 µL nuclease free water and further dilute with 210 µL of conjugation solution.

Note

Nuclease free water must be used, otherwise the oligonucleotide barcodes will be degraded.

9 Respective barcode solutions are added to the reduced primary antibodies and incubated for 02:00:00 at ~ 20 °C .

10 *Purify the solution by buffer exchanging with 450 µL purification solution. Centrifuge at 12000 x g for 00:08:00 . Discard flow-through.

11 go to step #10 twice.

12 Store in 100 µL antibody storage solution at 4 °C .

Tissue Preparation for CODEX Multiplexed Analysis

13 Remove fresh frozen tissue from -80 °C and place inside a desiccator for 00:20:00 .

14 Submerge tissues in excess acetone for 00:05:00 .

- 15 Rehydrate tissues in hydration buffer for 00:03:00 inside a humidity chamber.
 - 16 Fix the tissues for 00:10:00 in 1.6% paraformaldehyde in hydration buffer.
 - 17 Submerge tissues in excess staining buffer for 00:30:00.
 - 18 Incubate tissues with a blocking buffer and primary antibody cocktail (1:200 antibody dilution) for 03:00:00 within a humidity chamber at ~ 20 °C.
- Note

Tissues can be incubated in primary antibody solutions overnight if placed inside a refrigerator. Background may increase if longer incubation times are used but may be necessary for some antigen targets.
- 19 Rinse samples with staining buffer and fix with 1.6% paraformaldehyde in storage buffer for 00:10:00.
 - 20 Rinse with excess PBS and incubate in cold methanol for 00:05:00. Rinse samples with excess PBS again.
 - 21 Fix the samples with fixative solution for 00:10:00 before being stored in storage buffer at 4 °C.

Fluorescence Microscopy and CODEX Multiplexed Immunofluorescence

- 22 Dilute antibodies in reporter solution to a 1:200 dilution with 1 µL of a 1 mg/mL solution of DAPI or Hoechst 33342.
- Note

Most secondary oligonucleotide barcode reporters function at a 1:200 dilution; however, this dilution may need to be optimized in select cases or with custom reagents.
- 23 Fill respective bottles with nuclease free water, 1x CODEX buffer, and dimethyl sulfoxide.
 - 24 Mount a blank coverslip into the coverslip microfluidic chamber and flush the fluidic lines.
 - 25 Mount the prepared tissue sample and incubate with 1x CODEX buffer and 1 µL of 1 mg/mL of DAPI for 00:05:00 and wash with the automated CODEX system.
 - 26 Set up tiled regions covering the tissue and focus at the tissue surface.

- 27 Perform automated microscopy using a Zeiss Axio equipped with a Colibri 7 LED light source and C13440 camera or other equivalent equipment.

Note

Important parameters include a 10% tile overlap and a z-stack ranging from 11 to 20 tiles (depending on the tissue size).