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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.

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PROTOCOL CITATION

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KEYWORDS

CODEX, antibody conjugation, BIOMIC, HuBMAP, Vanderbilt University, multiplexed immunofluorescence

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GUIDELINES

Wear PPE and work in a well ventilated area.

MATERIALS TEXT

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** .

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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.

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.

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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** .

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.

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.

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

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

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