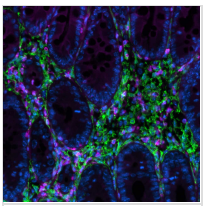

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Immunofluorescence on Formalin-Fixed Paraffin-Embedded Tissue Sections

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La Jolla Institute Microscopy Core

Katarzyna Dobaczewska

Fluorescence staining of FFPE tissues that leads to consistent and quality results.

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Make sure all reagents used in the Freequenza rack are at room temperature to avoid air bubble formation that can affect the quality of your stain.
For antigen retrieval citrate gives better signal to noise ratio in most cases. However, some antibodies will only work with Tris-EDTA buffer.

When first optimizing a new antibody run the following controls.
a. Unstained slide (Autofluorescence control)
b. Isotype control
c. Secondary only control
d. A couple of dilutions of your antibody.

0.1% Tween-20-containing 1XPBS solution **Contributed by users**

Prolong Gold **Thermo Fisher Scientific Catalog #P36930**

Pro-Par Clearant **Anatech LTP Catalog #510**

Hoechst 33342 **Contributed by users Catalog #H3570**

TrueBlack® Lipofuscin Autofluorescence Quencher **Biotium Catalog #23007**

Materials:
Reagents:
-Pro-par Clearant
-Reagent alcohol
-Antigen Retrieval Buffer: **Citrate (PH 6.0)** (10mM Sodium Citrate .05% Tween 20 pH 6.0) or **Tris-EDTA buffer(pH 9.0)**(10mM Tris Base 1mM EDTA .05% Tween 20 9.0)
-Wash Buffer: 1X PBS-T (.1% tween 20 detergent)(1ul/mL)
-Blocking solution : 5% normal serum (donkey) 0.3% triton X in PBS
-Primary antibody of choice
-Secondary antibody of choice
-Hoechst 33342
-True Black Autofluorescence Quencher
-Prolong gold

Lab equipment:
- **Epredia™ Shandon™ Plastic Coverplates** Cat no.72-110-017
- **Decloaking Chamber™ NxGen** with slide racks and metal slide canister
- **Freequenza**
3D design : https://3dprint.nih.gov/discover/3dpx-012172
Assembly: https://www.protocols.io/view/freequenza-box-production-and-assembly-byqmpvu6
-Coplin Jar

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Deparaffinization/ Rehydration

1 a. Bake slides for 1 hour at **60 °C**

b. Dip slides in Propar 20 times then let sit for 10 min. Repeat step 3x, each time with fresh Propar.

c. Dip slides in 100% reagent alcohol 20 times then let sit for 1 min 30 sec. Repeat step with fresh alcohol.

d. Dip slides in 90% reagent alcohol 20 times then let sit for 1 min 30 sec.

e. Place the slides under running DI water for 2 min.

Antigen Retrieval

2 a. Place slides in a rack into the Biocare Medical metal slide canister

b. Fill canister with Citrate antigen retrieval buffer.

b. Use the Biocare Medical decloaking chamber on program 5.

c. After program completion remove from chamber and let cool on the lab bench for 30 min.

d. Move rack into a fresh container, fill with PBS-T and let sit for 2 min.

Freequenza Setup

3 b. Cover slides with plastic Shandon coverplates and place them into the Freequenza rack.

c. Wash with 1mL of PBS-T and watch the flowrate through the Freequenza apparatus. If any coverplate slot appears to drain faster than the rest remove it from Freequenza rack and reapply the coverplate. If issue persists dispose of coverplate.

Place Slide on the feet at the bottom of the coverplate with the tissue facing towards the coverplate. Do this step under PBS (First portion of the video is to demonstrate what to do in the PBS buffer).

Check for air bubbles and that the slide is properly placed. If air bubbles are present redo this step.

Snap into place on the freequenza rack.

Check the flow rate.

Proceed with staining.

Blocking

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4 Place 100µL of blocking buffer (5% normal serum (donkey) 0.3% triton X in PBS) into coverplate slot and incubate for 1hr at **Room temperature**

Incubate with Primary Antibody

5 Place 100µL of optimal dilution of your purified antibody in PBS-T incubate at **4 °C** overnight.

Incubate with Secondary Antibody

6

Before proceeding with secondary antibody incubation let Freequenza rack equilibrate to room temperature for an hour.

a. Wash 4x with 1mL of PBS-T
b. Incubate with 100µL secondary antibody diluted to optimal concentration in PBS-T for 1hr at **Room temperature**
c. Wash 4x with 1mL of PBS

Counterstaining

7 a. Place 100uL of Hoechst 1:1000 (PBS) (10mg/mL stock) on each slide, incubate for 5 min.
b. Wash 3X with 1mL of PBS

Autofluorescence Quenching

8 a. Working in small batches remove slides from Freequenza and lay flat
b. Tap excess PBS off the slides
c. Overlay slides with TrueBlack Lipofuscin Autofluorescence Quencher (diluted 1:20 with 70% ethanol) incubate for approximately 30 seconds.
d. Place slides into a Coplin jar and fill with PBS. Let sit for 10 minutes.
e. Overfill the jar to allow residual TrueBlack to come to the top of the buffer surface. Discard the buffer from Coplin jar and refill it. Let sit for 10 min. Repeat step.

Coverslipping

9 Coverslip with 2-3 drops of ProLong Gold antifade mounting medium without DAPI

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Remove excess mounting media: Using a lab vacuum aspirator pull the excess from the edges of the cover glass.
Lay flat and allow to dry for at least 24 hours in the dark.

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