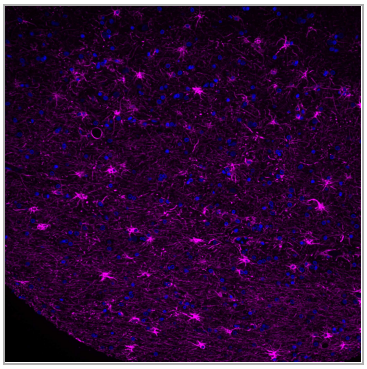

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**Protocol status:** Working
We use this protocol and it's working

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# Indirect immunofluorescence - tissue staining in TMA and whole tissue FFPE sections

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## ABSTRACT

Immunofluorescence staining allows detection and localization of antigens in different tissue types providing high sensitivity. The indirect immunofluorescence protocol is based on the principle of using a primary antibody binding to the target epitope, and a fluorophore-tagged secondary antibody that recognizes and binds to the primary antibody. This methods provides signal amplification allowing detection of several targets in the same tissue sample. This detailed protocol describes an adapted protocol, from An atlas of the protein-coding genes in the human, pig, and mouse brain article, for tissue staining in Tissue MicroArrays (TMA) and whole tissue Formalin Fixed Paraffin Embedded (FFPE) sections which is used in Emma Lundberg research group at Science for Life Laboratory; KTH - Royal Institute of Technology.

## MATERIALS

Product suggestion: Cancer Diagnostics, Inc.™ Moist Mark Plus™ Marking Pen, Fisher Scientific, cat# 22-500-210. It is an slide marker resistant to solvents, light and water resistant.
Product suggestion: EasyDip™ slide staining kit, Simport Scientific, cat# M906-12AS.
Product suggestion: TintoRetriever - heat retrieval system, Bio SB.
Product suggestion: 2 items of A4 Ultra bright LED light box pad 25.000 lux.
Product suggestion: Scienceware® Coplin staining jar with screw cap, Sigma, cat# S5641-12EA.
Product suggestion: PAP pen, Sigma, cat# ab2601.
Product suggestion: StainTray slide staining system, Sigma, cat# Z670146-1EA.
Product suggestion: Hoechst 33342, ThermoFisher Scientific, cat# H3570.
Product suggestion: Tris Buffered Saline (TBS), Medicago, cat# 09-7500-100.
Product suggestion: Tween-20, Sigma, cat# P1379.
Product suggestion: Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 555, ThermoFisher, cat# A21424. Suggested dilution: 1:800.
Product suggestion: Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 647, ThermoFisher, cat# A32728. Suggested dilution: 1:800.
Product suggestion: Rectangular cover glasses, VWR, cat# 631-0147.

## SAFETY WARNINGS

Refer to the SDS of each of the solvents and chemicals used in this protocol for safe lab practices. Consult your organization to learn the appropriate way to dispose the chemical waste.

## ETHICS STATEMENT

Review the ethical permits needed for the project and ensure to have all the documentation in place before starting any experiment.

The cerebral cortex tissue core used, to generate the thumbnail image of this protocol, was provided in the framework of a collaboration with Atlas Antibodies who acquired the tissue from BioIVT.

## BEFORE START INSTRUCTIONS

Review the protocol before starting it to ensure having all the material needed.

# Tissue preparation

1 Place the microscope slide (tissue facing up) in a slide warmer and bake it at 55 °C during 01:00:00. 1h

2 Label the slide. 2m

> Note
>
> **Product suggestion**: Cancer Diagnostics, Inc.™ Moist Mark Plus™ Marking Pen, Fisher Scientific, cat# 22-500-210. It is an slide marker resistant to solvents, light and water resistant.

3 Place the microscope slide in a staining rack and let it cool down for 00:05:00. 5m

4 Start the deparaffinization and hydration steps (Fig. 1): place the staining rack carefully in each of the next solvents, following the order, during 00:05:00. Ensure to close the lid of each of the containers to avoid evaporation.

Note

**Product suggestion**: EasyDip™ slide staining kit, Simport Scientific, cat# M906-12AS.


Fig. 1 | Slide staining station includes one anodized aluminum rack along with six assorted color jars (two white ones) and one slide staining rack. The aluminum holder can hold up to 6 staining jars. The anodized surface is resistant to rust, corrosion, and abrasion.

**4.1** HistoChoice Clearing agent **Merck MilliporeSigma (Sigma-Aldrich) Catalog #H2779 1L** 5m

**4.2** HistoChoice Clearing agent **Merck MilliporeSigma (Sigma-Aldrich) Catalog #H2779 1L** 5m

**4.3** Ethanol absolute ≥99.8% AnalaR NORMAPUR® ACS Reag. Ph. Eur. analytical reagent **VWR International Catalog #20821.330P** 5m

**4.4** Ethanol absolute ≥99.8% AnalaR NORMAPUR® ACS Reag. Ph. Eur. analytical reagent **VWR International Catalog #20821.330P** 5m

**4.5** 90% Ethanol prepared with ddH2O. 5m

**4.6** Meanwhile prepare the pressure cooker, to perform antigen retrieval step, filling it with ddH2O using the following settings: 106-110 °C and low pressure allowing to heat up. 5m

> Note
>
> **Product suggestion**: TintoRetriever - heat retrieval system, Bio SB.

**4.7** 70% Ethanol prepared with ddH2O. 5m

**4.8** 50% Ethanol prepared with ddH2O. 5m

**4.9** 30% Ethanol prepared with ddH2O. 5m

**4.10** ddH2O. 5m

**4.11** ddH2O. 5m

**4.12** Prepare the 1x citrate buffer solution in the container from the heat retrieval system: 25 ml Citrate Buffer pH 6.0 10× Antigen Retriever **Merck MilliporeSigma (Sigma-Aldrich) Catalog #C9999-1000ML** + 225 ml ddH2O. 5m

**4.13** Transfer the microscope slide to the staining rack fitting the container with the 1x citrate buffer and place the lid on top. 1m

**4.14** Place the covered container into the heat retrieval system and set up the following settings: 114-121 °C, high pressure for 00:20:00. 20m

**4.15** Remove the container from the heat retrieval system and allow to equilibrate to RT for at least 30min (otherwise tissue detachment may occur on the slide). 30m

**4.16** Transfer the microscope slide to a staining rack and place it in a container with ddH2O and leave it for 00:02:00 then transfer it to a second container with ddH2O for additional 00:02:00. 4m

## Optional: Photobleaching treatment

**5**

Note

**Original source**: Du et al. 2019. Nature Protocols 14: 2900-2930.

**Protocol modified by**: Derek Oldridge, M.D. Ph.D. and Jonathan Belman M.D. Ph.D.

Use two LED-lights to apply directly to the tissue to reduce the tissue autofluorescence. To avoid direct exposure to the lights, use a container (Fig. 2) and place inside the LED-lights (Fig.3) creating a sandwich where the sample will be located between them in a falcon tube.


Fig. 2 | Yellow plastic box suitable to store the LED-lights.

Note

**Product suggestion**: 2 items of A4 Ultra bright LED light box pad 25.000 lux.

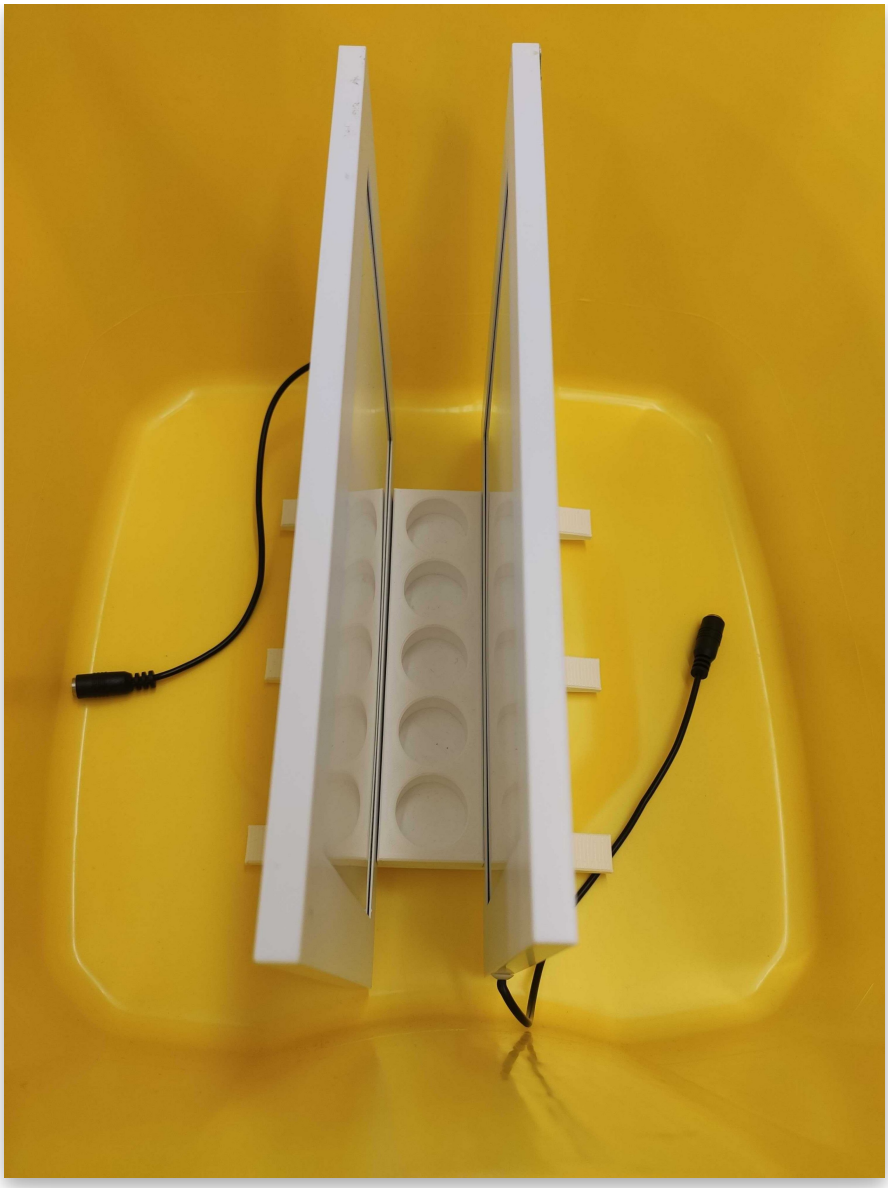

Fig. 3 | The photobleaching treatment may be performed inside the yellow plastic box to avoid direct light exposure.

6 Prepare the photobleaching solution in a 50 ml tube: 25 ml 1xPBS + 4.5 ml 30% (w/w) Hydrogen Peroxide Solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #31642-500ML** and 0.8 ml 1M NaOH. 5m

7 Transfer the microscope slide into the 50 ml tube containing the solution and place the tube in the rack between the LED-lights. Turn them on at maximum capacity during 00:45:00 . 45m

8 Depending of the type of tissue, the sample may undergo a second photobleaching incubation 50m

repeating go to step #6 and go to step #7.

9 Wash the sample with 1x PBS during 00:03:00. 3m

10 Repeat the for a total of 4 washes. 10m

## Staining day 1

11 Transfer the staining rack to a Coplin staining jar with 1x PBS: meanwhile, using a PAP pen, draw an hydrophobic barrier around the tissue. 5m

Note

**Product suggestion:** Scienceware® Coplin staining jar with screw cap, Sigma, cat# S5641-12EA.

Fig. 4 | Coplin jar with five internal slots that store up to 10 standard microscope slides. It made of opaque plastic.

Note

**Product suggestion:** PAP pen, Sigma, cat# ab2601.

12 Create an humidity chamber: fill the stainTray slide staining system (Fig. 5) with ddH2O to create 2m

a humidity environment to incubate the tissue with the 1ary antibodies.

Note

**Product suggestion**: StainTray slide staining system, Sigma, cat# Z670146-1EA.

Fig. 5 | Humidity chamber with black lid for tissue incubation.

**13** Prepare the Antibody Diluent solution (0.3 % Triton, 0.1 % NaN3 in 1x PBS pH = 7.4): add 30µl Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8787** + 10 µl of 10 % NaN3 in 10 ml 1x PBS pH = 7.4 in a falcon. 5m

**14** Prepare the Antibody Cocktail solution, diluting the 1ary antibody into the Antibody Diluent Solution to 100 µl as a final volume. 5m

**15** Take the microscope slide and remove the excess of water gently with a wipe without touching the tissue. 1m

**16** Using a suitable pipette: take 100 µl of the Antibody Cocktail solution, place the tip into a corner of the drawn hydrophobic barrier and slowly release the volume. The liquid will cover the tissue within the limits of the dawn barrier. 1m

Note

1. Avoid: tissue getting dried.
2. Avoid: liquid touching the hydrophobic barrier.

17 Incubate at 4 °C Overnight . 1d

## Staining day 2

2h 17m

18 Take an aliquot of TNB buffer and leave it at RT. 1m

Note

**TNB preparation suggestion**: 0.1 M Tris-HCl, pH 7.5; 0.15 M NaCl; 0.5% Blocking Reagent **Akoya Biosciences Catalog # SKU FP1020** . Hoechst (10 µl) may be added to perform already the nuclear staining during the blocking step.

Note

**Product suggestion**: Hoechst 33342, ThermoFisher Scientific, cat# H3570.

19 Fill a Coplin jar with TBS-T (TBS-0.1% Tween), remove the slide from the humidity chamber and place it in the Coplin jar: incubate for 00:15:00 . 16m

Note

**Product suggestion**: Tris Buffered Saline (TBS), Medicago, cat# 09-7500-100.
**Product suggestion**: Tween-20, Sigma, cat# P1379.

20 go to step #19 for a total of 3 washes. 35m

21 Take the microscope slide and remove the excess of water gently with a wipe without touching the tissue. Place it in the humidity chamber. 1m

22 Block the tissue adding 100 µl TNB buffer covering the tissue, incubate exactly 00:30:00 at Room temperature in the humidity chamber. 30m

23 Prepare 2ary antibody solution diluted in TNB to 100 µl as a final volume. 5m

Note

**Product suggestion**: Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 555, ThermoFisher, cat# A21424. Suggested dilution: 1:800.

**Product suggestion**: Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 647, ThermoFisher, cat# A32728. Suggested dilution: 1:800.

24 Remove the excess of water gently with a wipe without touching the tissue and place it again in the humidity chamber. 1m

25 Incubate the 2ary antibody adding 100 µl 2ary antibody solution covering the tissue, incubate 01:30:00 at Room temperature in the humidity chamber. 1h 30m

Note

**Critical!** Close the humidity chamber with the black lid.

26 Fill a Coplin jar with TBS-T (TBS-0.1% Tween), remove the slide from the humidity chamber and place it in the Coplin jar: incubate for 00:15:00. 15m

27 go to step #26 for a total of 3 washes. 35m

28 Take a coverslip suitable to cover the tissue and add the corresponding Fluoromount-G™ **Thermo Fisher Catalog #00-4958-02** volume (it may vary depending on the size of the coverslip). 2m

> Note
>
> **Product suggestion**: Rectangular cover glasses, VWR, cat# 631-0147.

29 Take the microscope slide and remove the excess of water gently with a wipe without touching the tissue. Place it on the bench and the take the coverslip with the mounting media to cover the tissue. 1m

30 Leave it to dry and, afterwards, seal the edges of the slide with nail polish (avoid adding to much as it may give autofluorescence). 15m

31 Store the mounted slide in a slide box, opaque lid, and keep at 4°C until performing the image acquisition. 1m