Dec 05, 2025

# Opal Multiplex Protocol

DOI
https://dx.doi.org/10.17504/protocols.io.x54v956rql3e/v1

Mariam Khaled[1,2], Ethan Vallebuona[3], Taryn Rauff[3], Debora Endo Colodete[3], Inna Smalley[3]

[1]The Department of Metabolism and Physiology, The Moffitt Cancer Center & Research Institute, Tampa, FL, USA;
[2]Department of Biochemistry, Faculty of Pharmacy, Cairo University, Egypt.;
[3]The Department of Metabolism and Physiology, The Moffitt Cancer Center & Research Institute, Tampa, FL, USA.

Mariam's Space

Mariam Lotfy Khaled
Cairo University, Moffitt Cancer Center





**DOI: https://dx.doi.org/10.17504/protocols.io.x54v956rql3e/v1**

**Protocol Citation:** Mariam Khaled, Ethan Vallebuona, Taryn Rauff, Debora Endo Colodete, Inna Smalley 2025. Opal Multiplex Protocol. **protocols.io https://dx.doi.org/10.17504/protocols.io.x54v956rql3e/v1**

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

**Created:** November 25, 2025

**Last Modified:** December 05, 2025

**Protocol Integer ID:** 233494

**Keywords:** opal multiplex protocol multiple immunofluorescence, staining mouse brain section, mouse tissue section, mouse brain section, different proteins on the same tissue, multiplex, same tissue, staining kit, akoya opal, different protein

## Abstract

Multiple immunofluorescence staining using the Akoya Opal staining kit requires multiple steps and precautions. In this protocol, I provide a detailed explanation of all the required steps for staining mouse brain sections. This protocol can be used to stain other human or mouse tissue sections. This multiplex staining is a cost-effective technique that enables the visualization of the spatial distribution of six different proteins on the same tissue.

## Guidelines

All mouse work was conducted in accordance with recognized ethical guidelines and in accordance with IACUC approval.

## Materials

1- EasyDip™ Slide Staining System, Simport Scientific (SMPM900-12AS)
2- Xylene
3- Ethanol
4- TBS (509013077, BioRad)
5- Tween (P7949-500ML, Sigma)
6- 10% Neutral Buffered Formalin
7- Cs IHC-Fencing Seal (A Type, R-CSC091-0, and B Type R-CSC092-01) from Nacalai USA.
8- Slide Staining Tray: Polypropylene (6PTJ1, Grainger)
9- Opal Anti-Rb HRP Kit (ARR1001KT), "Opal 6-Plex Manual Detection Kit for Whole Slide Imaging (NEL861001KT)
10- ProLong™ Diamond Antifade Mountant, 10 mL (P36970, Fisher)

## Troubleshooting

## Safety warnings

MAKE SURE TO COVER THE SLIDES THROUGHOUT THE WHOLE PROCESS

## Ethics statement

All mouse work and tissue collection were conducted according to IACUC protocol number IS00012698R

## Before start

Mouse brains were collected immediately after euthanasia.
The tissue was fixed in 10% neutral buffered formalin for ~ 24 hours, then transferred to aqueous 70% ethanol and stored at 4°C.

## Method

1 All brains (with or without skull) were fixed in 10% Neutral Buffered Formalin for 24 hours and then
transferred to 70% ethanol and kept at 4°C. Brains with skulls were decalcified by using 12.5 % EDTA (pH 7.5, adjusted using NaOH pellets) (BP120-1, Fisher) at 4°C for 7 days. EDTA was changed every 3 days. Then all tissues were transferred to 70% ethanol and kept at 4°C. All brains were sent to the Moffitt Histology Core for Paraffin embedding and sectioning at a thickness of 4 µm. All sections were scanned with the Akoya slide scanning microscope at Moffitt Cancer Center Microscopy Core. Images were visualized using Phenochart 1.2.0 software.

## Monoplex protocol

2 This step is to validate the antibody incubation conditions. Prepare a negative control slide.

3 Deparaffinization: immerse slide in Xylene, 2× 10 minutes

4 Rehydration: immerse slide in 100%, 95%, 70%, 50%Ethanol, distilled water for 1× 5 minutes each.

5 Antigen Retrieval (1hr step) (BioGenex, dilute 1:10 with DI water): Heat Antigen Retrieval Solution to boiling point in a Coplin jar (~60 seconds).

6 Add slides and leave in boiling buffer; re-warm every 5 minutes for 30 minutes (or continuous 30 minutes at 10% power). Leave it in the buffer until it cools (approximately 15-30 minutes).

7 Wash with water ddH2O for 5 minutes.

8 Seal the slide with a PAP pen or CS fencing seal (B type) Wipe any excess water with a Kimwipe

9 Blocking (30 min step): the slide with 150uL blocking (1% BSA, 2.5% Goat serum, 0.3% Triton X in PBS) for 30 minutes. No Wash between blocking and primary

10 Incubate with primary Ab antibody (1-hour step): Dilute the primary in the blocking solution. Add 150 µL to each slide and incubate at room temperature for 1 hour. Use a humidified box (with Wet tissue inside) and cover the slides to prevent them from drying. Cover the slides to avoid drying

11 Wash PBST (PBS+0.1% Triton X) or TBST (TBS+0.1% Tween) 3× 2 minutes, keep it on the rotor. Wipe any excess water with a Kimwipe

12 Incubate with the secondary Goat antirabbit (30 mins step). Use 2 µL (2mg/mL) with 1 mL blocking buffer to achieve a final concentration of 4 µg/mL. Add 100 µL of secondary on the section in the dark container (humidified chamber). Cover the slides to avoid drying

13 Wash PBST (TBST) 3× 2 minutes, keep it on the rotor. Wipe any excess water with a Kimwipe

14 Remove the Pap pen with a cotton swap containing xylene, and clean it with a Kim wipe (or remove the fencing seal)

15 Mount coverslips (use Prolong Antifade with DAPI for fluorescent stains). Keep the slides at 4 °C until the imaging.

## Dropout Staining Protocol

16 Choose 6 sections. Prepare TBST (1X TBS + 0.1% Tween20)

17 Deparaffinization. Place the slides in a Coplin jar in the bead bath at 65 °C for 4 hours to allow the paraffin to melt (or 56 °C overnight). The next day, place the slides in the grey slide holder (Fig. 1A) and drop them into the xylene jar for 10 minutes, twice. Hydrate through an ethanol gradient for 5 minutes each (100%, 95%, 70%, 50%), followed by a 5-minute incubation in distilled water (Fig. 1B).

18 Slide Fixation. Fill a Jar with 10% Neutral Buffered Formalin and drop the slide holder into the 10% NBF for 20 minutes to fix the tissue. Follow this with a distilled water wash (immerse the slide holder in the Jar with distilled water for 5 minutes).

19 Antigen Retrieval/MWT. Fill a jar with 1X AR 6 solution, then boil it in the microwave at 100% power (Fig. 1C, D). Next, place the slide holders (containing the slides) in the jar and adjust the microwave to 30 minutes at 10% power. Allow the slides to cool to room temperature on the bench for at least 15 minutes. Remove the Antibody Diluent/Blocking from the refrigerator to allow it to come to room temperature while the AR6 is cooling.

20 Blocking. Wash with deionized water (ddH2O) for 5 minutes (Fig. 1E). Wipe the extra solution with a Kimwipe (Fig. 1F). Add the IHC-Fencing Seal around the tissue section (Fig. 1G). Add 3-5 drops of Antibody Diluent/Blocking (Fig. 2A, B) and incubate the tissue for 10 min at room temperature (RT). Prepare the Primary dilution during the waiting and return the Antibody Diluent/Blocking to the Fridge.

21 Primary Antibody Incubation. Remove the blocking solution by tapping the slides on the Bench working pad. Spin down the primary antibody tube, prepare an adequate dilution using Antibody Diluent/Blocking, and spin down the Mixture (Fig. 2C, D). Then, apply 100-150 µL of the target protein antibody for one hour. (Open the Nozzle of the Diluent bottle). Prepare the secondary before washing to allow it to come to room temperature. Wash slides with 1X TBST for 3 times, each for 2 minutes on the Rotor (Fig. 3 A-C).

22 Secondary Antibody Incubation. Mix Opal Polymer HRP secondary antibody ARH2001EA with Opal Polymer anti-Rabbit Diluent (1:4) and spin it down to mix it. Then apply 100-150 µL of secondary working solution to the tissue section, then incubate for 10 min at RT (Fig. 3D, E). Remove the OPAL and the Amplification solution from the fridge to allow them to come to room temperature while waiting. Wash slides with 1X TBST for 3 times, 2 min each.

23 Opal Fluorophore Incubation. Prepare a 1:100 dilution of Opal of the target protein working solution. For each slide, mix 1 µL of Opal with 100 µL of the Amplification solution, spin down the mixture, then apply 100- 150 µL to the tissue and incubate for 10 min at RT (Fig. 3 F-H).

24 Remove IHC-Fencing Seal.

25 Microwave Treatment (MWT). Fill a jar with 1X AR 6 solution, then boil it in the microwave at 100%. Next, place the slide holders (containing the slides) in the jar and adjust the microwave to 15 minutes at 10% power. Allow slides to cool down to room temperature on the bench for at least 15 min.

26 Secondary Antibody Incubation. Mix Opal Polymer HRP secondary antibody ARH2001EA with Opal Polymer anti-Rabbit Diluent (1:4) and spin it down to mix it. Then apply 100-150 µL of secondary working
solution to the tissue section, then incubate for 10 min at RT.

27 Wash slides with 1X TBST for 3 times, 2 min each.

28 Opal Fluorophore Incubation. Prepare a 1:100 dilution of the next Opal working solution. For each slide, mix 1 µL of Opal with 100 µL of the Amplification solution, spin down the mixture, then apply 100- 150 µL to the tissue and incubate for 10 min at RT. Wash slides with 1X TBST 3 times, 2 minutes each.

29 Spectral DAPI. Add two drops of DAPI solution to 1 mL of TBS. Discard any unused portion of DAPI Working Solution. Rinse slides in distilled water and then in TBST. Add 150 µL of DAPI Working Solution to each slide. Incubate the slides in the DAPI solution for 5 minutes at room temperature.

30 Wash the slides with TBST for 2 minutes, followed by 2 minutes in distilled water.

31 Mount. Apply mounting medium (ProLong® Diamond Antifade Mountant) for fluorescence microscopy and coverslip.

## Multiple staining of six markers

32 MAKE SURE TO COVER THE SLIDES THROUGHOUT THE WHOLE PROCESS

33 Cycle 1:

33.1 Antigen Retrieval/MWT. Fill a jar with 1X AR 6 solution, then boil it in the microwave at 100% power. Next, place the slide holders (containing the slides) in the jar and adjust the microwave to 30 minutes at 10% power. Allow the slides to cool to room temperature on the bench for at least 15 minutes. Remove the Antibody Diluent/Blocking from the refrigerator to allow it to come to room temperature while the AR6 is cooling.

33.2 Blocking. Wash with deionized water (ddH2O) for 5 minutes. Wipe the extra solution with a Kimwipe. Add the IHC-Fencing Seal around the tissue section. Add 3-5 drops of Antibody Diluent/Blocking and incubate the tissue for 10 min at room temperature (RT). Prepare the Primary dilution during the waiting and return the Antibody Diluent/Blocking to the Fridge.

33.3 Primary Antibody Incubation. Remove the blocking solution by tapping the slides on the Bench working pad. Spin down the primary Ab tube, prepare an adequate dilution using Antibody Diluent/Blocking, and spin down the Mixture. Then, apply 100-150 µL of anti-GFAP (1:100) antibody for one hour. (Open the Nozzle of the Diluent bottle). Prepare the secondary before washing to allow it to come to room temperature.
Wash slides with 1X TBST for 3 times, each for 2 minutes on the Rotor.

33.4 Secondary Antibody Incubation. Mix Opal Polymer HRP secondary antibody ARH2001EA with Opal Polymer anti-Rabbit Diluent (1:4) and spin it down to mix it. Then apply 100-150 µL of secondary working solution to the tissue section, then incubate for 10 min at RT. For two slides, I prepared a 50uL + 150uL solution. Remove the OPAL and the

Amplification solution from the fridge to allow them to come to room temperature while waiting.
Wash slides with 1X TBST for 3 times, 2 min each.

33.5 Opal Fluorophore Incubation. Prepare a 1:100 dilution of Opal-620 working solution. For each slide, mix 1 µL of Opal with 100 µL of the Amplification solution, spin down the mixture, then apply 100-150 µL to the tissue and incubate for 10 min at RT. DO NOT FORGET THE SLIDE COVERING
Wash slides with 1X TBST 3 times, 2 minutes each.
Remove IHC-Fencing Seal

33.6 Microwave Treatment (MWT). Fill a jar with 1X AR 6 solution, then boil it in the microwave. Next, place the slide holders (containing the slides) in the jar and adjust the microwave to 15 minutes at 10% power. Allow the slides to cool down to room temperature on the bench for at least 15 min. Remove the Antibody Diluent/Blocking from the fridge to allow it to come to room temperature while the AR6 is cooling.

34 Cycle 2:

34.1 Blocking. Wash with deionized water (ddH2O) for 5 minutes. Wipe the extra solution with a Kimwipe. Add the IHC-Fencing Seal around the tissue section. Add 3-5 drops of Antibody Diluent/Blocking and incubate the tissue for 10 min at room temperature (RT). Prepare the Primary dilution during the waiting and return the Antibody Diluent/Blocking to the Fridge.

34.2 Primary Antibody Incubation. Remove the blocking solution by tapping the slides on the Bench working pad. Spin down the primary Ab tube, prepare an adequate dilution using Antibody Diluent/Blocking, and spin down the Mixture. Then, apply 100-150 µL of anti-CD163 (1:100) antibody for one hour. For 2 slides, I used 2 µL of antibody in 200 µL of diluent (Open the Nozzle of the Diluent bottle). Prepare the secondary before washing to allow it to come to room temperature.
Wash slides with 1X TBST for 3 times, each for 2 minutes on the Rotor. DO NOT FORGET THE SLIDE COVERING

34.3 Secondary Antibody Incubation. Mix Opal Polymer HRP secondary antibody ARH2001EA with Opal Polymer anti-Rabbit Diluent (1:4) and spin it down to mix it. Then apply 100-150 µL of secondary working solution to the tissue section, then incubate for 10 min at RT. For two slides, I prepared a 50uL + 200uL solution. Remove the OPAL and the Amplification solution from the fridge to allow them to come to room temperature while waiting.
Wash slides with 1X TBST for 3 times, 2 min each.

34.4 Opal Fluorophore Incubation. Prepare a 1:100 dilution of Opal-520 working solution. For each slide, mix 1 µL of Opal with 100 µL of the Amplification solution, spin down the mixture, then apply 100-150 µL to the tissue and incubate for 10 min at RT.

Wash slides with 1X TBST 3 times, 2 minutes each.
Remove IHC-Fencing Seal

34.5 Microwave Treatment (MWT). Fill a jar with 1X AR 6 solution, then boil it in the microwave at 100% power. Next, place the slide holders (containing the slides) in the jar and adjust the microwave to 15 minutes at 10% power. Allow the slides to cool to room temperature on the bench for at least 15 minutes. Remove the Antibody Diluent/Blocking from the fridge to allow it to come to room temperature while the AR6 is cooling.

35 Cycle 3:

35.1 Blocking. Wash with deionized water (ddH2O) for 5 minutes. Wipe the extra solution with a Kimwipe. Add the IHC-Fencing Seal around the tissue section. Add 3-5 drops of Antibody Diluent/Blocking and incubate the tissue for 10 min at room temperature (RT). Prepare the Primary dilution during the waiting and return the Antibody Diluent/Blocking to the Fridge.

35.2 Primary Antibody Incubation. Remove the blocking solution by tapping the slides on the Bench working pad. Spin down the primary Ab tube, prepare an adequate dilution using Antibody Diluent/Blocking, and spin down the Mixture. Then, apply 100-150 µL of anti-CD8 (1:100) antibody for one hour. For 2 slides, I used 2 µL of antibody in 200 µL of diluent (Open the Nozzle of the Diluent bottle). Prepare the secondary before washing to allow it to come to room temperature.
Wash slides with 1X TBST for 3 times, each for 2 minutes on the Rotor. DO NOT FORGET THE SLIDE COVERING

35.3 Secondary Antibody Incubation. Mix Opal Polymer HRP secondary antibody ARH2001EA with Opal Polymer anti-Rabbit Diluent (1:4) and spin it down to mix it. Then apply 100-150 µL of secondary working solution to the tissue section, then incubate for 10 min at RT. For two slides, I prepared a 50uL + 200uL solution. Remove the OPAL and the Amplification solution from the fridge to allow them to come to room temperature while waiting.
Wash slides with 1X TBST for 3 times, 2 min each.

35.4 Opal Fluorophore Incubation. Prepare a 1:100 dilution of Opal-480 working solution. For each slide, mix 1 µL of Opal with 100 µL of the Amplification solution, spin down the mixture, then apply 100-150 µL to the tissue and incubate for 10 min at RT.
Wash slides with 1X TBST 3 times, 2 minutes each.
Remove IHC-Fencing Seal

35.5 Microwave Treatment (MWT). Fill a jar with 1X AR 6 solution, then boil it in the microwave at 100% power. Next, place the slide holders (containing the slides) in the jar and adjust the microwave to 15 minutes at 10% power. Allow the slides to cool to room temperature on the bench for at least 15 minutes. Remove the Antibody Diluent/Blocking from the refrigerator to allow it to come to room temperature while the AR6 is cooling.

36 Cycle 4:

36.1 Blocking. Wash with deionized water (ddH2O) for 5 minutes. Wipe the extra solution with a Kimwipe. Add the IHC-Fencing Seal around the tissue section. Add 3-5 drops of Antibody Diluent/Blocking and incubate the tissue for 10 min at room temperature (RT). Prepare the Primary dilution during the waiting and return the Antibody Diluent/Blocking to the Fridge.

36.2 Primary Antibody Incubation. Remove the blocking solution by tapping the slides on the Bench working pad. Spin down the primary Ab tube, prepare an adequate dilution using Antibody Diluent/Blocking, and spin down the Mixture. Then, apply 100-150 µL of anti-CD8 (1:100) antibody for one hour. For 2 slides, I used 2 µL of antibody in 200 µL of diluent (Open the Nozzle of the Diluent bottle). Prepare the secondary before washing to allow it to come to room temperature.
Wash slides with 1X TBST for 3 times, each for 2 minutes on the Rotor. DO NOT FORGET THE SLIDE COVERING

36.3 Secondary Antibody Incubation. Mix Opal Polymer HRP secondary antibody ARH2001EA with Opal Polymer anti-Rabbit Diluent (1:4) and spin it down to mix it. Then apply 100-150 µL of secondary working solution to the tissue section, then incubate for 10 min at RT.
For two slides, I prepared a 50uL + 200uL solution. Remove the OPAL and the Amplification solution from the fridge to allow them to come to room temperature while waiting.
Wash slides with 1X TBST for 3 times, 2 min each.

36.4 Opal Fluorophore Incubation. Prepare a 1:100 dilution of Opal-480 working solution. For each slide, mix 1 µL of Opal with 100 µL of the Amplification solution, spin down the mixture, then apply 100-150 µL to the tissue and incubate for 10 min at RT.
Wash slides with 1X TBST 3 times, 2 minutes each.
Remove IHC-Fencing Seal

36.5 Microwave Treatment (MWT). Fill a jar with 1X AR 6 solution, then boil it in the microwave at 100% power. Next, place the slide holders (containing the slides) in the jar and adjust the microwave to 15 minutes at 10% power. Allow the slides to cool to room temperature on the bench for at least 15 minutes. Remove the Antibody Diluent/Blocking from the refrigerator to allow it to come to room temperature while the AR6 is cooling.

37 Cycle 5:

37.1 Blocking. Wash with deionized water (ddH2O) for 5 minutes. Wipe the extra solution with a Kimwipe. Add the IHC-Fencing Seal around the tissue section. Add 3-5 drops of Antibody Diluent/Blocking and incubate the tissue for 10 min at room temperature (RT).

Prepare the Primary dilution during the waiting and return the Antibody Diluent/Blocking to the Fridge.

37.2 Primary Antibody Incubation. Remove the blocking solution by tapping the slides on the Bench working pad. Spin down the primary Ab tube, prepare an adequate dilution using Antibody Diluent/Blocking, and spin down the Mixture. Then, apply 100-150 µL of anti-MBP (1:200) antibody for one hour. For 2 slides, I used 2.5 µL of antibody in 500 µL of diluent (spin it down to mix it) (Open the Nozzle of the Diluent bottle instead of the dropper). Prepare the secondary before washing to allow it to come to room temperature.
Wash slides with 1X TBST for 3 times, each for 2 minutes on the Rotor. DO NOT FORGET THE SLIDE COVERING

37.3 Secondary Antibody Incubation. Mix Opal Polymer HRP secondary antibody ARH2001EA with Opal Polymer anti-Rabbit Diluent (1:4) and spin it down to mix it. Then apply 100-150 µL of secondary working solution to the tissue section, then incubate for 10 min at RT. For two slides, I prepared a 50uL + 200uL solution. Remove the OPAL and the Amplification solution from the fridge to allow them to come to room temperature while waiting.
Wash slides with 1X TBST for 3 times, 2 min each.

37.4 Opal Fluorophore Incubation. Prepare a 1:100 dilution of Opal-570 working solution. For each slide, mix 1 µL of Opal with 100 µL of the Amplification solution, spin down the mixture, then apply 100-150 µL to the tissue and incubate for 10 min at RT.
Wash slides with 1X TBST 3 times, 2 minutes each.
Remove IHC-Fencing Seal

37.5 Microwave Treatment (MWT). Fill a jar with 1X AR 6 solution, then boil it in the microwave at 100% power. Next, place the slide holders (containing the slides) in the jar and adjust the microwave to 15 minutes at 10% power. Allow the slides to cool to room temperature on the bench for at least 15 minutes. Remove the Antibody Diluent/Blocking from the fridge to allow it to come to room temperature while the AR6 is cooling.

38 Cycle 6:

38.1 Blocking. Wash with deionized water (ddH2O) for 5 minutes. Wipe the extra solution with a Kimwipe. Add the IHC-Fencing Seal around the tissue section. Add 3-5 drops of Antibody Diluent/Blocking and incubate the tissue for 10 min at room temperature (RT). Prepare the Primary dilution during the waiting, and return the Antibody Diluent/Blocking to the Fridge.

38.2 Primary Antibody Incubation. Remove the blocking solution by tapping the slides on the Bench working pad. Spin down the primary Ab tube, prepare an adequate dilution using Antibody Diluent/Blocking, and spin down the Mixture. Then, apply 100-150 µL of anti-

MAP2 (1:200) antibody for one hour. (Open the Nozzle of the Diluent bottle). Prepare the secondary before washing to allow it to come to room temperature.
Wash slides with 1X TBST for 3 times, each for 2 minutes on the Rotor. DO NOT FORGET THE SLIDE COVERING

38.3 Secondary Antibody Incubation. Mix Opal Polymer HRP secondary antibody ARH2001EA with Opal Polymer anti-Rabbit Diluent (1:4) and spin it down to mix it. Then apply 100-150 µL of secondary working solution to the tissue section, then incubate for 10 min at RT.
For two slides, I prepared a 50uL + 200uL solution. Remove the OPAL and the Amplification solution from the fridge to allow them to come to room temperature while waiting.
Wash slides with 1X TBST for 3 times, 2 min each.

38.4 Opal Fluorophore Incubation. Prepare a 1:100 dilution of Opal-690 working solution. For each slide, mix 1 µL of Opal with 100 µL of the Amplification solution, spin down the mixture, then apply 100-150 µL to the tissue and incubate for 10 min at RT.
Wash slides with 1X TBST 3 times, 2 minutes each.
Remove IHC-Fencing Seal

38.5 Microwave Treatment (MWT). Fill a jar with 1X AR 6 solution, then boil it in the microwave at 100% power. Next, place the slide holders (containing the slides) in the jar and adjust the microwave to 15 minutes at 10% power. Allow the slides to cool to room temperature on the bench for at least 15 minutes. Remove the Antibody Diluent/Blocking from the fridge to allow it to come to room temperature while the AR6 is cooling.

38.6 Opal Fluorophore 780 Incubation. Wash with deionized water (ddH2O) for 5 minutes. Prepare a 1:25 dilution of Opal-780 working solution. For each slide, mix 4 µL of Opal with 100 µL of the Diluent/Blocking (NOT the amplification Sol.), spin down the mixture, then apply 100-150 µL to the tissue and incubate for 10 min at RT.
Wash slides with 1X TBST 3 times, 2 minutes each.

39 Spectral DAPI. Add two drops of DAPI solution to 1 mL of TBS. Discard any unused portion of DAPI Working Solution.
Rinse slides in distilled water and then in TBST. Add 150 µL of DAPI Working Solution to each slide. Incubate the slides in the DAPI solution for 5 minutes at room temperature.
Wash the slides with TBST for 2 minutes, followed by 2 minutes in distilled water.

40 Mount. Apply mounting medium (ProLong® Diamond Antifade Mountant) for fluorescence microscopy and coverslip.

## Results

41 Result: All the Opal Fluorophores dropout staining were clear, showing different protein targets (Fig. 4). Additionally, the multiplex staining using brain with skull showed all the Opal fluorophores (Fig. 5).

## Figure 1

42 Figure 1

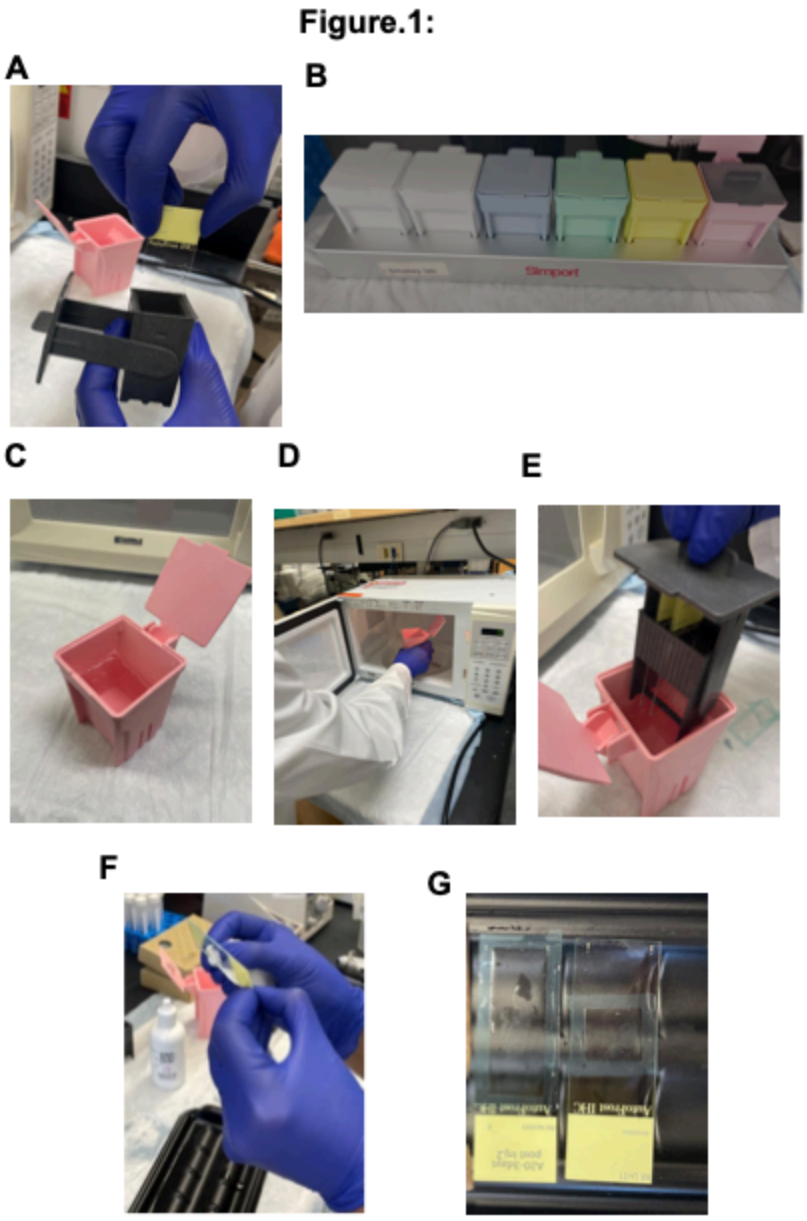


Figure 1

## Figure 2

43 Figure 2

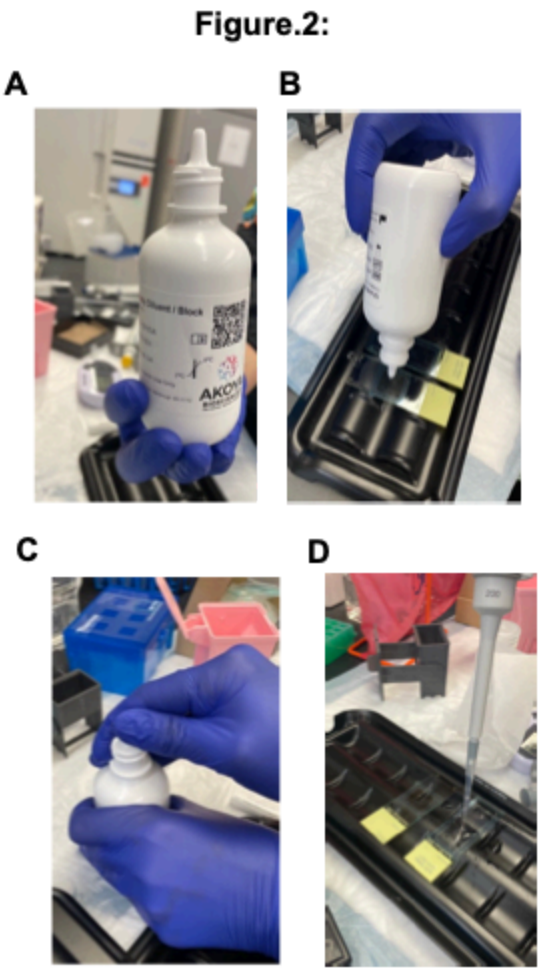


Figure 2

## Figure 3

44 Figure 3

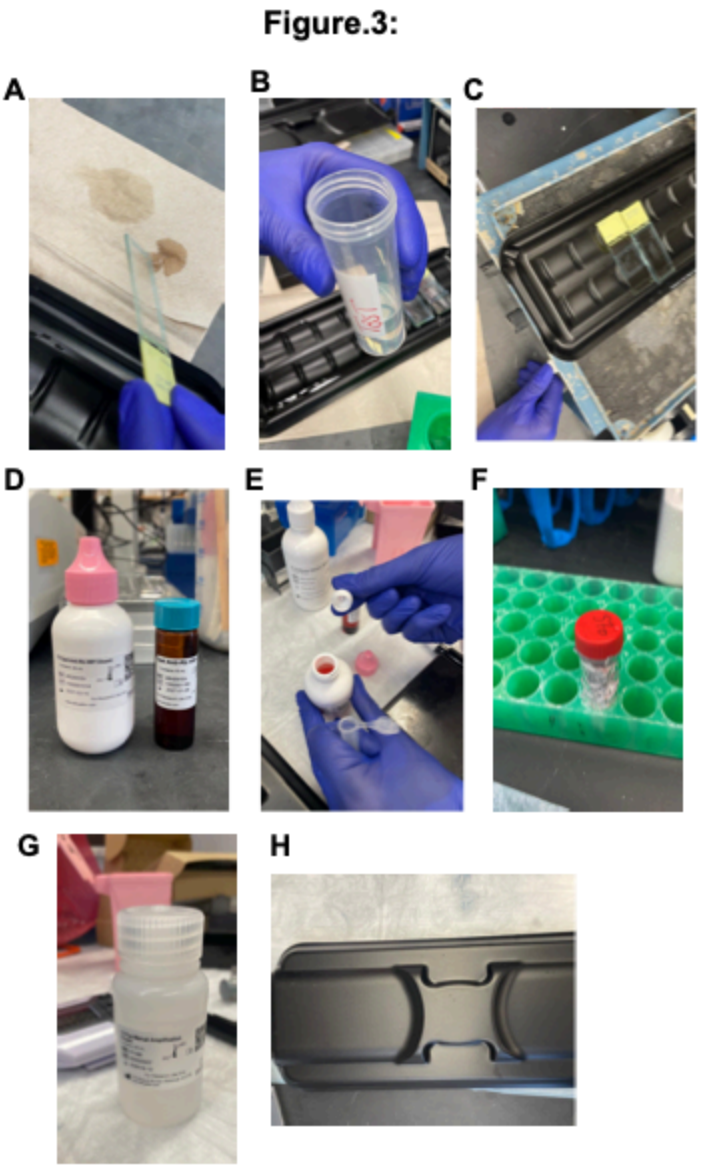


Figure 3

## Figure 4

45 Figure 4

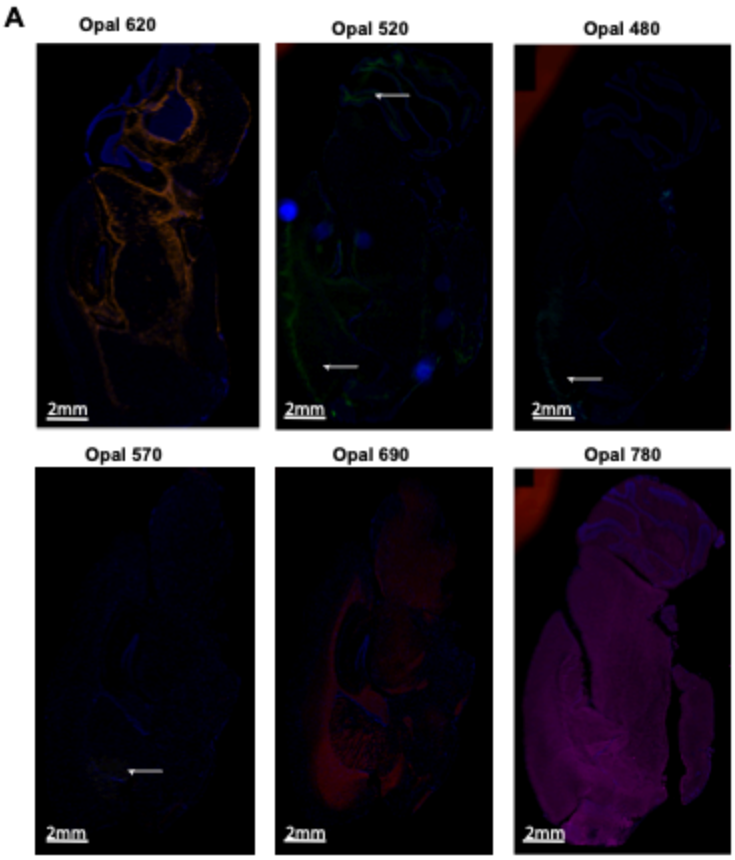


Figure.4: Dropout Opal staining: We used the following antibody-fluorophore combinations: GFAP (Cell Signaling Technology, 80788S, 1:100) with opal 620 fluorophore (1:100), CD163 (abcam, ab182422, 1:100) with opal 520 fluorophore (1:100), CD8+ (Cell Signaling Technology, 98941S, 1:100) with opal 480 fluorophore (1:100), Iba1/AIF-1 (Cell Signaling Technology, 17198S, 1:100) with opal 570 fluorophore (1:100), MBP (Cell Signaling Technology, 78896S , 1:200) with opal 690 fluorophore (1:100) and MAP2 (Cell Signaling Technology, 8707S, 1:200) with opal 780 fluorophore (1:100)

Figure 4

## Figure 5

46

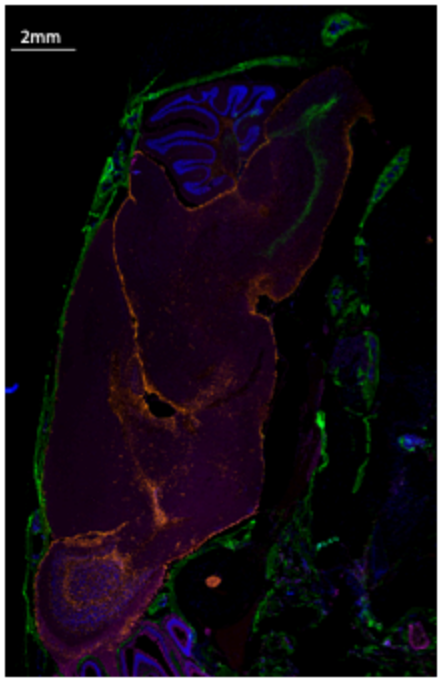


Figure.5: Multiplex Opal staining: Brain with skull was staineed with different Opal flourophores in the following order. GFAP (Cell Signaling Technology, 80788S, 1:100) with opal 620 fluorophore (1:100), CD163 (abcam, ab182422, 1:100) with opal 520 fluorophore (1:100), CD8+ (Cell Signaling Technology, 98941S, 1:100) with opal 480 fluorophore (1:100), Iba1/AIF-1 (Cell Signaling Technology, 17198S, 1:100) with opal 570 fluorophore (1:100), MBP (Cell Signaling Technology, 78896S , 1:200) with opal 690 fluorophore (1:100) and MAP2 (Cell Signaling Technology, 8707S, 1:200) with opal 780 fluorophore (1:100)

Figure 5

## Protocol references

https://view-su2.highspot.com/viewer/644694a9965a147858092f49?source=email.644694a9965a147858092f4a.32

## Acknowledgements

Joseph Johnson, Director of Microscopy Core at Moffitt Cancer Center