Dec 15, 2025

4i (iterative indirect immunofluorescence imaging) Protocol for FFPE slides - Benchmarking project Sennet

DOI
https://dx.doi.org/10.17504/protocols.io.e6nvw4xqwlmk/v1

Nicola Neretti[1], Samantha Madnick[1]

[1]Brown University

Cellular Senescence Net...

Samantha Madnick
Brown University

DOI: https://dx.doi.org/10.17504/protocols.io.e6nvw4xqwlmk/v1

Protocol Citation: Nicola Neretti, Samantha Madnick 2025. 4i (iterative indirect immunofluorescence imaging) Protocol for FFPE slides - Benchmarking project Sennet. **protocols.io** https://dx.doi.org/10.17504/protocols.io.e6nvw4xqwlmk/v1

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

Created: December 11, 2025

Last Modified: December 15, 2025

Protocol Integer ID: 234814

Keywords: iterative indirect immunofluorescence imaging (4i), FFPE, tissue section, antibody elution, protein, using iterative indirect immunofluorescence imaging, iterative indirect immunofluorescence imaging, indirect immunofluorescence imaging, eluting antibodies between imaging round, multiplexed protein measurement, protein targets within the same tissue section, protocol for ffpe slide, imaging round, ffpe slide, imaging, protein target, eluting antibody, secondary antibody, antibody, ffpe, benchmarking project sennet

Abstract

This protocol describes a workflow for highly multiplexed protein measurements using iterative indirect immunofluorescence imaging (4i). This method is optimized for formalin-fixed paraffin-embedded (FFPE) tissue sections and enables repeated cycles of staining, imaging and antibody elution using conventional primary and secondary antibodies. By sequentially eluting antibodies between imaging rounds, this approach allows the detection of a large number of protein targets within the same tissue section while preserving spatial context. This protocol was adapted from methods developed in the laboratory of Dr Joao Passos.

Materials

- Ice
- SuperFrost Plus slides
- Histoclear
- Ethanol (100%, 90%, 70%)
- Distilled water
- Coplin jars
- Deionized water
- Citrate buffer 1x working solution
- 10x Citrate buffer pH 6.0 (Abcam AB64214)
- Hot plate or microwave
- Heat resistant gloves
- Aluminum foil
- PBS
- 5% Bovine serum albumin (BSA)
- 2.5M maleimide stock
- DMSO
- H2O2 (Sigma-Aldrich #216763)
- NaOH
- LED lamps
- 6-well plate

Safety warnings

- Use heat resistant gloves when handling boiling solutions.
- Aliquot in fume hood.

Sectioning of paraffin blocks

1 Samples are placed on ice for at least 20 minutes

2 Trim block until sample is exposed

3 Sections are cut at 5um

4 Sections are laid on a water bath that is between 40-45˚C to unfold wrinkles

5 Sections are picked up onto a SuperFrost Plus slide

6 Slides are air dried at room temperature for 30 minutes

7 Slides are placed in an oven at 60˚C overnight

Deparaffinization and Hydration

8 Deparaffinize and hydrate tissue sections through Histoclear 2×5 min (in fume hood), 100% ethanol 2×5 min, 90% ethanol 5 min, 70% ethanol 5 min, distilled water 2×5 min. These steps are performed in Coplin Jars

9 Steamer assembly

9.1 Fill the water basin with deionized water

9.2 Place the plastic steaming compartment atop the water basin

9.3 Place an empty Coplin jar in the steaming compartment

9.4 Turn on the steamer until steam is visible escaping the vent. Keep the lid closed

9.5 Using a hot plate or microwave, boil ~75 ml of citrate buffer 1x working solution

9.6 Citrate buffer 1x working solution
7ml 10x Citrate buffer pH 6.0 (Abcam AB64214)
Add 63 ml distilled water

9.7 Using heat resistant gloves, carefully pour the boiling sodium citrate buffer into Coplin jar within the steaming compartment

9.8 Add slides to Coplin jar, cover in aluminum foil and close lid

10 Incubate for 10 minutes

11 Turn off steamer and remove lid. Make sure citrate buffer solution is still covering entire tissue

12 Let slides to cool down in the citrate buffer 20-30 minutes at room temperature (or to cool it faster place on ice until buffer is cold)

13 Wash sections in distilled water 2×5 min

14 Wash sections for 5 min PBS on shaker

4i staining protocol

15 Surround sections with hydrophobic pen, add 4i blocking buffer and place in humidified chamber. Incubate for 1 hr RT (first cycle 1h, next cycles 20 min)

15.1 4i blocking buffer (5mL)
740 ul 1x PBS
200 ul 5% Bovine serum albumin (BSA) (1% BSA)
60 ul 2.5M maleimide stock (150mM maleimide)

Use within 48h
Store at 4˚C

15.2 2.5M maleimide stock solution
Dissolve 1g maleimide in 2mL DMSO. Bring to 4mL total volume with DMSO.
Make 200 uL aliquots and store at -20C.
Aliquot in fume hood.

15.3 5% BSA
0.5 g into 10 ml PBS
Store 4˚C and use within a week

16 Wash sections for 2×5 min PBS on shaker

17 Lung only: Prepare the Bleaching solution in a 50mL centrifuge tube. Final working solution: 4.5% (w/v) H2O2 and 20mM NaOH in PBS. Submerge the slides in the Bleaching solution using the lid of a 6-well plate. Sandwich the 6-well plate between the two LED lamps (https://a.co/d/8ciGKlU) for 45 minutes at room temperature.

17.1 Bleaching Solution
25 ml 1x PBS
4.5 ml 30% (wt/vol) H2O2 (Sigma-Aldrich #216763)
0.8 ml 1M NaOH

18 Wash the tissues four times in 1x PBS for 3-5 minutes per wash.

19 Add primary antibody cocktail diluted in conventional blocking buffer overnight 4˚C.

19.1 Conventional blocking buffer (500L)
400ul 1x PBS
100 ul 5% Bovine serum albumin (BSA) (1% BSA)

20 Wash sections for 2×5 min PBS on shaker

21 Add secondary antibody cocktail diluted in conventional blocking buffer for 2h RT.

22 Wash sections for 5 min PBS on shaker

23 Incubate with DAPI 1:1000 in PBS for 10 min RT.

24 Wash sections for 2×5 min PBS on shaker

25 Add 75 µl of imaging buffer to section and carefully place coverslip on top. Wipe excess of liquid around coverslip and make sure coverslip is attached to slide.

25.1 Imaging buffer (3 mL)
342mg n-acetylcysteine (store at 4˚C)
450uL H2O
Dissolve and bring pH to 7.5 with ~1.95ml 1M NaOH.
Bring final volume to 3mL with H2O.
Make fresh daily and keep cold

26 Conduct imaging using confocal microscope

27 Dip sections in a Coplin jar filled with PBS and gently shake to remove coverslip.

28 Add elution buffer to sections. Incubate 10 minutes on shaker in humidified box. Repeat 4x with fresh buffer.

28.1 2x Elution buffer without TCEP (50mL)
3.75g L-glycine (0.5M)
18g urea (3M)
Put weighed L-glycine and urea into an empty 50 ml tube. The powder volume will fill almost the entire tube.
Add 10 ml H2O and mix to dissolve most of the glycerin and urea to make space for the guanidine hydrochloride
28.66g guanidine HCl (3M)
Vortex to dissolve or mix on rotation
Adjust volume to 50 ml with H2O
Store 4C for several months

28.2 Elution Buffer
700 ul H2O
1.217 ml 2x Elution Buffer
333 ul TCEP (open fresh aliquot and aliquot the rest and store -20˚C)
Adjust the pH to 2.5 by mixing in ~400 ul 6N HCl

Make Fresh each day

29 Repeat protocol starting with blocking (20 minutes) on step 1. Do not repeat antigen retrieval or bleaching step (step 3)