🔬 Immunofluorescence (IF) Staining

Fluorescent Antibody Detection Protocol

Protocol Overview

Immunofluorescence (IF) is a technique that uses fluorescently-labeled antibodies to detect specific proteins or antigens in tissue sections. This protocol is designed for both paraffin-embedded and cryosectioned tissue samples.

⏱️ Duration

Day 1: ~3 hours (+ overnight incubation)

Day 2: ~2 hours

Total: 2 days

🎯 Key Steps

1. Deparaffinization/Antigen retrieval
2. Blocking (1 hour)
3. Primary antibody (overnight)
4. Secondary antibody (1 hour)
5. DAPI and mounting

> 💡 **Important Notes:**
>
> - Protect slides from light after adding fluorescent antibodies
> - Work quickly during mounting to prevent drying

- Store stained slides at 4°C protected from light
- Image slides within 1-2 weeks for best results

Required Materials

Reagents

- Xylene (for paraffin sections)
- 100%, 95%, 70%, 50%, 20% Ethanol
- ddH_2O (distilled water)
- Citrate antigen retrieval buffer

  Solution A: 0.1M citric acid
  Solution B: 0.1M sodium citrate
- PBS-T (PBS + 0.1% Triton X-100)
- 10% serum (matched to secondary antibody host)
- Primary antibody
- Secondary antibody (fluorescent conjugate)
- DAPI (1 mg/mL stock)
- Fluoromount mounting medium

Equipment

- Coplin jars
- Microwave
- Humidifying chamber (can use wet paper towels in closed box)
- PAP pen (hydrophobic barrier pen)
- Coverslips
- Timer
- 4°C refrigerator or cold room
- Slide storage box (opaque, light-protected)
- Pipettes and tips

Solution Recipes

10% Serum Block:
100 µL serum + 900 µL PBS-T

2% Serum (Antibody Dilution):
20 µL serum + 980 µL PBS-T

DAPI Working Solution:
1 µL DAPI (1 mg/mL) + 2 mL PBS-T (1:2000 dilution)

Select Protocol Day:

Day 1 (3 hours + overnight) | Day 2 (2 hours)

Day 1: Deparaffinization, Antigen Retrieval, and Primary Antibody

Total time: ~3 hours + overnight incubation

Select Sample Type:

Paraffin Sections | Cryo Sections

1 Deparaffinization (~30 minutes)

- [ ] Xylene - 10 minutes
- [ ] Xylene - 3 minutes
- [ ] Xylene - 3 minutes
- [ ] 100% EtOH - 2 minutes
- [ ] 100% EtOH - 2 minutes

- [ ] 95% EtOH - 1 minute
- [ ] 70% EtOH - 1 minute
- [ ] 50% EtOH - 1 minute
- [ ] 20% EtOH - 1 minute
- [ ] Transfer to ddH_2O

2 Antigen Retrieval (~1 hour)

- [ ] Place slides in coplin jar with citrate buffer, sit 10 min at RT
  ⚠️ Have a second jar with buffer only to microwave alongside slides
- [ ] Microwave 2.5 min at 50% power (power level 5)
- [ ] Microwave 2.5 min at power level 4
- [ ] Microwave 2.5 min at power level 4
- [ ] Microwave 2.5 min at power level 4
- [ ] Remove lid and allow to cool AT LEAST 30 minutes
  ⚠️ Critical: Do not rush cooling step
- [ ] Wash slides with PBS-T 3× for 5 min each

1 Antigen Retrieval (~15 minutes)

- [ ] Microwave citrate buffer 2.5 min at power level 5

- [ ] Pour heated buffer over slides
- [ ] Microwave slides 2.5 min at power level 4
- [ ] Allow to cool for 5 minutes
- [ ] Wash slides with PBS-T 3× for 5 min each

3 Blocking (1 hour)

- [ ] Prepare 10% serum in PBS-T (100 µL serum + 900 µL PBS-T)
  💡 Use serum from same species as secondary antibody host
- [ ] Incubate slides with 10% serum for 1 hour at room temperature

4 Primary Antibody (Overnight)

- [ ] Wash slides with PBS-T 5 min 3×
- [ ] Dilute primary antibody in 2% serum (20 µL serum + 980 µL PBS-T)
  💡 Dilution ratio varies by antibody - check datasheet
- [ ] Apply primary antibody to slides in humidifying chamber
- [ ] Incubate overnight at 4°C in dark
  ⚠️ Ensure humidifying chamber is well-sealed

Day 2: Secondary Antibody, DAPI, and Mounting

Total time: ~2 hours

1 Secondary Antibody (1 hour)

- [ ] **Wash slides with PBS-T 5 min 3×**
- [ ] **Dilute secondary antibody in 2% serum**
  💡 Common dilution: 1:200-1:500, check datasheet
- [ ] **Incubate slides for 1 hour at room temperature in dark**
  ⚠️ Protect from light - fluorophores are light-sensitive

2 DAPI Nuclear Staining

- [ ] **Wash slides with PBS-T 5 min 2×**
- [ ] **Dilute DAPI 1:2000 (1 µL of 1 mg/mL stock into 2 mL PBS-T)**
- [ ] **Incubate slides in DAPI for 1 minute**

3 Mounting

- [ ] **Wash slides with PBS-T 5 min 2×**
- [ ] **Add Fluoromount mounting medium**
  💡 Use aqueous mounting medium - do NOT use xylene-based mounting medium
- [ ] **Place coverslip gently and remove air bubbles**

- [ ] **Store slides at 4°C protected from light**
  ⚠️ Image within 1-2 weeks for optimal fluorescence

Imaging Tips

Minimize Photobleaching

Keep exposure times as short as possible and reduce light intensity when finding focal plane. Work quickly to prevent fluorophore degradation under light exposure.

Optimal Timing

Image slides within 1-2 weeks of staining for best fluorescence intensity. Some fluorophores fade over time even when stored properly.

Channel Selection

Image DAPI first (blue channel), then move to longer wavelengths (green, then red). This minimizes photobleaching of more sensitive fluorophores.

Control Slides

Always include: (1) No primary antibody control, (2) Isotype control, (3) Positive control tissue. These help validate specificity and optimize imaging settings.

Mounting Medium Curing

Allow mounting medium to cure for at least 24 hours at 4°C before imaging for optimal clarity. Imaging too soon may result in poor image quality.

Troubleshooting Guide

High Background Signal

Possible Causes:

- Insufficient blocking
- Antibody concentration too high
- Inadequate washing
- Tissue autofluorescence

Solutions:

- Increase blocking time or serum concentration
- Optimize antibody dilution (start with higher dilutions)
- Extend wash times and increase number of washes
- Use autofluorescence quencher or image at different wavelengths

No Signal or Weak Signal

Possible Causes:

- Antibody concentration too low
- Primary or secondary antibody not working
- Antigen not present or masked
- Photobleaching during imaging

Solutions:

- Optimize antibody dilution (use less dilution)
- Test antibodies with positive control tissue
- Optimize antigen retrieval method and duration
- Reduce light exposure during imaging

Non-Specific Staining

Possible Causes:

- Cross-reactivity of antibodies
- Secondary antibody binding non-specifically
- Endogenous biotin (if using biotin-based detection)

Solutions:

- Include no-primary antibody control
- Use isotype control to assess non-specific binding

- Pre-absorb antibody with tissue powder
- Switch to non-biotin detection system

Rapid Photobleaching

Possible Causes:

- Fluorophore is light-sensitive
- High light intensity during imaging
- Long exposure times

Solutions:

- Use more photostable fluorophores (Alexa Fluor series)
- Reduce light intensity and exposure time
- Use anti-fade mounting medium
- Image immediately after mounting

Tissue Fell Off Slides

Possible Causes:

- Slides not positively charged
- Microwave power too high
- Excessive agitation during washes

Solutions:

- Use positively charged slides only
- Reduce microwave power during antigen retrieval
- Be gentle during washing steps
- Bake slides at 60°C for 30-60 min before staining

⚠️

Safety Information

- Always work with xylene in a fume hood (for paraffin sections)
- Wear appropriate PPE (lab coat, gloves, safety glasses)

- Handle antibodies with care - some may contain sodium azide preservative
- Dispose of fluorescent waste appropriately
- Be cautious with hot liquids during antigen retrieval
- Follow proper storage conditions for antibodies (typically 4°C or -20°C)

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About This Protocol

Immunofluorescence (IF) staining protocol for detecting specific proteins using fluorescently-labeled antibodies in tissue sections.

Quick Links

Protocol Overview

Troubleshooting

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