Multiplexing Immunofluorescence

Using complexes pre-formed from primary antibodies and secondary fluorophores.
For research use only - not intended for diagnostic use.

Multiplexing immunofluorescence allows researchers to stain a single sample with multiple primary antibodies originating from the same species, even if they have identical isotypes. Stainings can, e.g., be performed with multiple monoclonal mouse IgG1 primary antibodies or with multiple antibodies raised in rabbit at the same time.

Abcam offers a choice of secondary fluorophore reagents that are suitable for multiplexing applications, see page 2.

All secondary fluorophore reagents offered are based on monovalent single-domain antibodies ("nanobodies") that are site-specifically coupled to two fluorophores per nanobody. They are supplied as solutions containing 5 µM nanobody (10 µM fluorophore).

Important general remarks

For multiplexing applications, all primary antibodies need to be well characterized. The stoichiometry between primary antibody and the secondary fluorophore reagent is critical. Therefore, the concentration of the primary antibody needs to be precisely known.

- Monoclonal antibodies are generally provided by the supplier at a defined concentration. In case cell culture supernatants are used or the concentration of the antibody is unknown, it might be required to purify the antibody e.g. via Protein G to remove serum proteins or other additives before quantification.
- For polyclonal antibodies, an affinity purification step is mandatory in order to remove serum proteins and antibodies not recognizing the target protein.

Components required

Multiplexing with primary mouse monoclonal antibodies

- Primary mouse monoclonal antibodies (not supplied)
- Fluorescently labeled secondary fluorophores matching the isotype of the primary antibodies used.

Multiplexing with affinity-purified primary rabbit antibodies

- Affinity-purified primary antibodies raised in rabbit (not supplied)
- Fluorescently labeled secondary fluorophore anti-Rabbit IgG

Protocol

1. Complex formation

1.1 Determine the amount of purified primary antibody required for your experiment. If unknown, start with 1 µg of each primary antibody.
1.2 To saturate all binding sites, combine in separate reaction tubes each of your primary antibodies with an appropriate labeled secondary fluorophore. Use 20 pmol (4 µL of secondary reagent as supplied by Abcam) of secondary fluorophore per µg primary antibody. This will result in a 50% excess of labeled product over available binding sites.
1.3 Add 20 µL PBS per µg of primary antibody
1.4 Incubate for 20 min at room temperature (RT) with moderate shaking.

2. Preparing the final staining solution

2.1 Mix and dilute all pre-formed complexes to reach the respective final concentrations required for your experiment. A recommended dilution buffer for immunofluorescence applications is PBS, 0.1 % Triton-X100, 1 % BSA.
2.2 Immediately proceed to step 3.

3 Staining procedure

∆***Note:*** *The optimal staining procedure may depend on the sample type and the primary antibodies used. We can therefore only give a starting protocol here that generally works well for standard immunofluorescence (IF) applications on fixed cultured cells.* Please see our other more detailed protocol for *immunofluorescence attached in the protocol section on the datasheet of the secondary fluorophore reagent.*

3.1 Stain your fixed and permeabilized sample for 1 h at RT protected from light.
3.2 Wash 3 x for 5 min with PBS.
3.3 Rinse samples with water before mounting to remove excess salt.
3.4 Mount and image sample.

Hints for optimization

- Test single stainings (without multiplexing) in order to determine the minimal concentration of primary secondary mixture required to give adequate staining with minimal background.
- Use the lowest concentrations of primary antibody and secondary tools required for optimal stainings. High concentrations of primary antibodies and/or large excess of secondary tools may result in non-specific background staining.
- Avoid long incubation and/or extensive washing steps whenever possible.
- For extended incubation steps include an appropriate multiplexing blocker reagent.
- It is ideal to image the samples shortly after mounting.

∆***Note:*** *An additional post-fixation step is recommended when encountering problems with fluorophore hopping after mounting the sample.*

List of secondary reagents

AB325286	Alpaca monoclonal [10A4] Anti-Mouse IgG1 antibody (AZdye 568)
AB325287	Alpaca monoclonal [10A4] Anti-Mouse IgG1 antibody (Alexa Fluor® 647)
AB325288	Alpaca monoclonal [10A4] Anti-Mouse IgG1 antibody (ATTO 488)
AB325292	Alpaca monoclonal [10E10] Anti-Rabbit IgG antibody (AZdye 568)
AB325293	Alpaca monoclonal [10E10] Anti-Rabbit IgG antibody (Alexa Fluor® 647)
AB325294	Alpaca monoclonal [10E10] Anti-Rabbit IgG antibody (ATTO 488)
AB325298	Alpaca monoclonal [14A4] Anti-Mouse IgG2a/b antibody (AZdye 568)
AB325299	Alpaca monoclonal [14A4] Anti-Mouse IgG2a/b antibody (Alexa Fluor® 647)
AB325300	Alpaca monoclonal [14A4] Anti-Mouse IgG2a/b antibody (ATTO 488)

For technical support contact information, visit: www.abcam.com/contactus

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