Multiplexing immunofluorescence with pre-formed primary/secondary antibody complexes

Introduction

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

NanoTag Biotechnologies offers a choice of secondary tools that are validated for multiplexing applications:

FluoTag®-X2 anti-Mouse IgG kLC (Order no. N1202)
FluoTag®-X2 anti-Mouse IgG1 (Order no. N2002)
FluoTag®-X2 anti-Mouse IgG2 (Order no. N2702)
FluoTag®-X2 anti-Rabbit IgG (Order no. N2402)

All secondary FluoTag®-X2 reagents offered by NanoTag are based on monovalent single-domain antibodies ("nanobodies") carrying two fluorophores each. They are supplied as solutions containing 5 µM nanobody (10 µM fluorophore).

General remarks

Primary antibody requirements

For multiplexing applications, all primary antibodies need to be well characterized and of high quality. As the stoichiometry between primary antibody and the secondary FluoTag reagent is critical, the concentration of the primary antibody needs to be known precisely. Monoclonal antibodies therefore need to be purified e.g. via Protein A to remove serum proteins. Affinity purification is mandatory for polyclonal tools in order to remove serum proteins and antibodies not recognizing the target protein.

Recommended trial experiments

Multiplexing in general is a delicate application, which may require optimization. We strongly recommend to first establish conventional single-color staining using secondary FluoTag reagents in order to get a feeling for the specific properties, blocking requirements and optimal concentration range of the individual primary antibodies used.

For further information concerning this protocol please contact us at

info@nano-tag.com

NanoTag Biotechnologies

Multiplexing Immunofluorescence

Procedure

For recommendations regarding sample preparation please see our general IF protocol.

1. Thoroughly quantify all primary antibodies.
2. In separate tubes, add a **2.5- to 3-fold excess of FluoTag-X2 reagent** to each primary antibody. This will result in a 1.25- to 1.5-fold excess of FluoTag-X2 reagent over available binding sites considering that there are two identical binding sites for the FluoTag reagent on each primary antibody.

   E.g., add 4 µL FluoTag-X2 reagent (= 20 pmol; stock concentration 5 µM nanobody) per microgram of primary antibody (1 µg of primary antibody = 6.7 pmol; M_r(IgG) = 150 kDa).
3. Dilute the mixture 5-fold with PBS and incubate for 1 h at RT with subtle shaking
4. Dilute pre-incubated primary/secondary complexes in established blocking buffer (we recommend 3% natural goat serum + 0.1% TX-100 in PBS) to reach concentrations optimized for each primary antibody before.
5. Stain your sample for 1 h at RT protected from light.
6. Wash 3x for 5 min with PBS (protected from light).
7. Before mounting, briefly rinse with water to remove excess of salt.
8. Image sample

Hints for optimization

1. Test **single stainings** (without multiplexing) in order to determine the minimal concentration of primary-secondary mixture required to give adequate staining with minimal background. Avoid using higher concentrations than necessary – excess antibody may account for non-specific background staining.
2. If available: After step 3 add an 2- to 5-fold excess of non-specific (control) IgG or serum of non-immunized animals to catch the excess of secondary FluoTag reagent.
3. After mixing the pre-formed complexes make sure to keep all incubation and washing steps as short as possible to prevent "hopping" of the secondary tools between different primary antibodies.
4. After step 6 an optional **post-fixation step** can be implemented (compatibility with fluorophores used needs to be tested)
   - Incubate with 4% PFA in PBS for 20 min at room temperature.
   - Wash once with quenching solution (100 mM NH_4Cl in PBS)
   - Incubate with quenching solution for 10 min at room temperature.
   - Wash 3x for 5 min with PBS (protected from light).
   - Proceed with step 7 above.

Not recommended

1. Over-night (or even longer) incubations of pre-formed complexes on the sample.
2. Using a large excess of secondary FluoTag-X2 reagent
3. Extensive washing (e.g. washing over night)
4. Prolonged storage before imaging

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