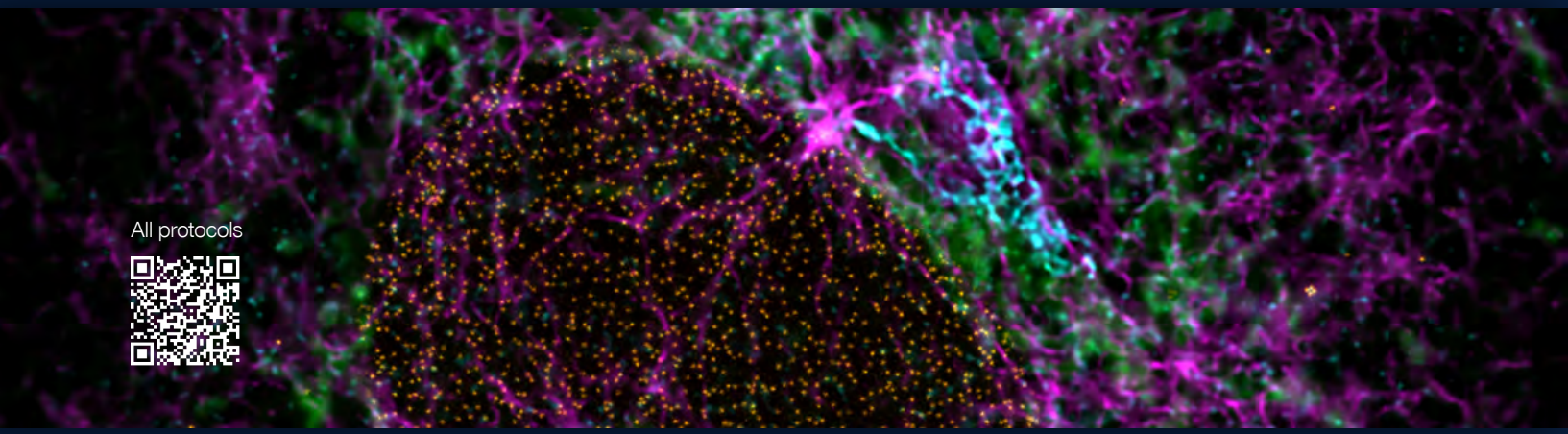


All protocols

Premix & stain protocol

for easy and proper application of our labels

Multiplexing immunofluorescence allows researchers to stain a single sample with multiple primary antibodies originating from the same species, even if they have identical isotype.

We offer a choice of secondary reagents that are validated for multiplexing applications:

- FluoTag®-X2 anti-Mouse IgG1
- FluoTag®-X2 anti-Rabbit IgG

Scan the QR code to discover more details on FluoTag®-X2 monoclonal secondary nanobodies!

Introduction

The combination of super bright and highly photostable *abberior* dyes and FluoTag®-X2 monoclonal secondary nanobodies (Smart Secondaries®) from NanoTag Biotechnologies offers a fantastic solution for high-resolution imaging experiments where small labels, good penetration, and outstanding specificity are necessary.

Storage & reconstitution

FluoTag®-X2 monoclonal secondary nanobodies (Smart Secondaries®)

All fluorescent FluoTag®-X2 monoclonal secondary nanobodies are lyophilized from PBS pH 7.4 with 2 % BSA (US-Origin) and shipped as lyophilized powder at ambient temperature. The lyophilized reagent can be stored at 2-8°C for up to 12 months.

Before usage, reconstitute and aliquot the reagent according to the detailed protocol below. After reconstitution in 500 µl, the final concentration is 10 µM dye and 5 µM nanobody.

- Prepare sterile 50% glycerol (v/v) in deionized water. If applicable, we recommend including 0.1% sodium azide as a preservative. Sodium azide should be avoided when staining live cells or conducting in vivo studies.
- Open the vial containing the lyophilized FluoTag® secondary reagent and add 500 µL of sterile 50 % glycerol (v/v) in deionized water.
- Mix gently and allow to sit at room temperature for approximately 5 min.
- Optional: Briefly spin down the vial for 2 min at 100 x g using a 50 ml conical tube with tissue paper at the bottom.
- Distribute into aliquots. Use small tubes and avoid aliquots below 20 µl.
- Storage: Working aliquot can be stored at -20°C for up to 4 weeks. Ideally store at -80°C (up to 6 months).

Notes: Avoid for all nanobody products repeated freeze-thaw cycles. Therefore, it is recommended to split the abberior secondary nanobody solution into smaller aliquots. Minimize exposure to light to prevent photobleaching of the dye.

Premix & stain protocol for cultured cells

For very fast and easy labeling, premix a primary antibody with the monoclonal nanobodies and stain your biological sample in just a few steps. This premix & stain approach allows for species independent multiplexing.

The procedure has been successfully tested with our abberior dye conjugates for adherent cells grown on glass coverslips and has yielded consistent results in most instances, but may require further optimization for particular model organisms or experimental conditions.

Required reagents; not provided

- Phosphate-buffered saline pH 7.4 (PBS)
- Fixative depending on the primary antibody
- 0.1% – 0.5% Triton X-100 in PBS pH 7.4 (permeabilization buffer)
- 2 % – 4 % Bovine Serum Albumin + 0.1% Tween 20 in PBS pH 7.4 (blocking buffer, PBT)
- Mounting Medium
- Glass coverslips with a glass thickness of ~170 µm (No.1.5 or No. 1.5H)

  Note: We do not recommend using plastic coverslips because frequently only suboptimal imaging results areachieved. If possible, coverslips with grids, gratings, orsimilar should be avoided, as these structures can interfere with imaging causing aberrations that degrade image quality.
- Humid chamber
- Fluorescence microscope with suitable excitation light source and detection filter

Protocol for cultured cells

All steps are carried out at room temperature and in a petri dish unless stated otherwise.

1. Incubate cells with a fixative suitable for the primary antibody in use for 5 min to 30 min.

   Note: When using Methanol as a fixative, step 2 can be skipped.

   Optional: Quench with PBS supplemented with 0.1 M glycine or 0.1 M NH4Cl for 10 min at RT.

2. Replace the solution with a permeabilization buffer and incubate the sample for 5 min to 15 min.

3. Wash cells 3 x 5 min in PBS.

4. Replace the PBS with a blocking buffer and incubate the sample for 30 to 60 min.

5. Premixing of primary antibodies and corresponding nanobody occurs in a small volume of 10 - 20 µl. For each premix consisting of a primary antibody and a secondary nanobody, a separate preparation is made in a reaction tube.

   - Please review the important general remarks regarding primary antibodies on page 3.

   - Determine the amount of purified primary antibody. If unknown, start with 1 µg of each primary antibody.

   - To saturate all binding sites, combine in separate reaction tubes each of your primary antibodies with an appropriate labeled FluoTag®-X2 monoclonal secondary nanobody. **Use 20 pmol (4 µl secondary reagent) secondary FluoTag® per µg primary antibody.** This will result in 50 % excess of labeled nanobody over available binding sites.

   - Add 10 - 20 µl PBS per µg of primary antibody.

   - Incubate for 20 min.

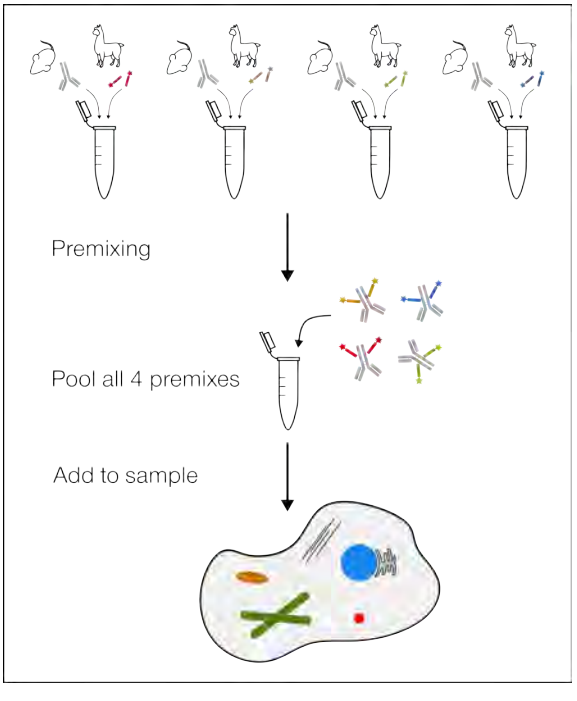
   Note: The molar ratios of primary antibody and FluoTag®-X2 monoclonal secondary nanobody mentioned here are meant as a starting point. The ratio of primary antibody to secondary nanobody depends on factors such as the target protein/ structure, dye, and primary antibody. The ideal ratio must therefore be determined individually for each experiment.

7. After incubation time, mix and dilute all preformed complexes with PBT to reach the final concentrations required for your experiment.

8. Take the cover slips out of the petri dish; remove the excess blocking buffer by placing the cover slip edge onto a piece of tissue paper. Transfer the coverslips into a humid chamber, cells facing upwards. Add the solution with the antibody-nanobody complexes onto the coverslips and incubate for 20 min to 60 min in the humid chamber under the exclusion of light.

9. Remove excess antibody-nanobody complex by placing the cover slip edge onto a piece of tissue paper. Wash the cells in PBS (3 x 5 min) using a fresh petri dish.

10. Take the cover slip out of the washing solution, remove excess PBS by placing the cover slip edge onto a piece of tissue paper, and mount the coverslip with a suitable mounting medium.



Important general remarks

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

- Monoclonal mouse 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 rabbit antibodies, an affinity purification step is mandatory in order to remove serum proteins and antibodies not recognizing the target protein

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. Add 10 µL of MXB-M or MXB-R (NanoTag Biotechnologies) per 1 µg of primary antibody to each individual premix after completing step 5. Incubate for 5 minutes before proceeding to step 6.
- It is ideal to image the samples shortly after mounting. An additional post-fixation step is recommended when encountering problems with nanobody hopping after mounting the sample.