MULTIPLEX IMMUNOFLUORESCENCE PROTOCOL

Multiplex immunofluorescence is used for simultaneous detection of several target proteins in the same cell. Primary antibodies should ideally be from different species, but in some cases it is possible to use antibodies raised in the same species. In the latter case, it is possible to use e.g. primary antibodies of different isotypes, which can then be visualised using subtype-specific secondary antibodies. The secondary antibodies should be from the same species (to prevent cross-reactivity). Many companies have special antibodies with minimal cross-reactivity done especially for co-localisation studies. Moreover, several control experiments must be performed to ensure the specificity of co-localisation of different targets (see below).

MATERIALS NEEDED

- Cryo- or paraffin-embedded sections mounted on SuperFrost slides.
- Target retrieval solutions pH6 or pH9 (if needed)
- Incubation chamber with wet Wettex stripes
- DAKO Cytomation pen
- 1xPBS
- TBS or TNT wash buffer
- Primary and secondary antibodies diluted in 1x PBS, containing 0.3% Triton, 0.01% NaAzide, 0.02% Bacitracin and BSA
- Fluorophore-conjugated secondary antibodies for regular immunofluorescence
- Mounting media (e.g. ProLong Gold with or without DAPI)
- Coverslips
- Eppendorf tubes for dilution of antibodies
- Pipettes and pipette-tips

PRIMARY ANTIBODY PREPARATION

- Pre-incubate primary antibody with BSA (0.5%) prior to application to the tissue.
- Dilute primary antibody in antibody diluent to a working concentration.

CRYO SECTIONS

- **Perfusion-fixed,** 10-30% sucrose cryoprotected sections cut in a microtome at 14 µm, thaw-mounted on Super Frost slides. Dry sections on slides for additional 2 h at room temperature (RT). Rehydrate in 1xPBS 2x15 min, or proceed with antigen retrieval.
- **Snap-frozen** sections cut in a microtome at 14 µm, thaw-mounted on Super-Frost slides and dried for 2h. Pre-fix sections in ice-cold paraformaldehyde (4%PFA) for 20 min (put frozen sections directly into 4%PFA, do not allow thawing). Rinse in 1x PBS 15 min.

ANTIGEN RETRIEVAL FOR CRYO-SECTIONS (OPTIONAL)

If target requires antigen retrieval, standard target retrieval solutions can be used, but the temperature and time should be reduced.

HEAT-INDUCED EPITOPE RETRIEVAL

1. HIER buffer pH 6, a modified citrate buffer, 60-70°C, 10 min.
2. HEIR buffer pH 9, a Tris/EDTA buffer, 60-70°C, 10 min.

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PARAFFIN-EMBEDDED TISSUE

Perfusion- or immersion-fixed paraffin embedded sections cut at 4 µm thick, mounted on Super Frost slides, dried at 50°C overnight.

IF ANTIGEN RETRIEVAL IS NOT REQUIRED

- Deparaffinise sections in xylene (2x5 min)
- Rehydrate sections:
  100% EtOH 2x3 min,
  95% EtOH 2x3 min,
  70% EtOH 2x3 min,
  rinse in ddH20
- Proceed with IHC procedure.

ANTIGEN RETRIEVAL FOR PARAFFIN SECTIONS (COMMON)

HEAT-INDUCED EPITOPE RETRIEVAL

1. HIER buffer pH6, a modified citrate buffer, 97°C, 20 min.
2. HIER buffer pH 9, a Tris/EDTA buffer, 97°C, 20 min.

Cool down with ddH2O, rinse in 1x wash buffer 15 min, and proceed with IHC.

IHC PROCEDURE

DAY I

1. Draw a circle around each section with DAKO Cytomation pen.
2. Rinse slides in 1x wash buffer for 15 min
3. **Blocking endogenous peroxidase:**
4. **Cryo sections**: Put slides in 0.03-0.1% H2O2 in 1xPBS for 5 min at RT to quench endogenous peroxidase activity
5. **Paraffin sections:** Put slides in 0.3% H2O2 in 1xPBS for 15 min at RT to quench endogenous peroxidase-activity
6. Rinse in PBS 15 min at RT
7. Block sections in 2% normal serum of the secondary antibody host, in PBS 30 min at RT (optional)
8. Add primary antibodies (diluted in 1x PBS, contai-ning 0.3% Triton, 0.01% NaAzide, 0.02% Bacitra-cin), approx. 180µl/slide. Optimal concentration is defined by user.
9. If primary antibodies are of different isotypes (IgG1, IgG2a, IgG2b), they can be mixed together.
10. Incubate in a humidified chamber overnight at 4ºC.

DAY II (IF-IHC)

11. Rinse slides in 1x wash buffer for 3x5 min at RT
12. Incubate with secondary antibodies conjugated with fluorophores (1:80 -1:800 in PBS), 30 min at 37ºC or 1 h at RT in the dark
13. Isotype-specific secondary antibodies can be mixed in the same incubation solution
14. Wash in wash buffer for 2x10 min at RT in the dark
15. Mount in anti-fading solution (e.g. ProLong Gold), let cure for 24 h at 4ºC
16. Ready for microscopy/scanning
17. Store at 4ºC.

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CONTROL PROTOCOLS

ADSORPTION

For each primary antibody an adsorption control should be done on single-stained sections:

- Incubate primary antibody (in working dilution which will be used on section next day) with corresponding peptide 10-6M overnight at 4ºC in an Eppendorf tube pre-coated with Sigmacote (trimetylcylan)
- Apply absorbed antibody on sections as in normal protocol Day I for overnight incubation, proceed as for IHC Day II.

TISSUE-IgG INTERACTIONS

- Omitting the primary antibody should result in blank sections.

SINGLE-STAIN CONTROL

The immunoreactivity observed on slides with co-labelling, should be identical with expression profile on adjacent single-stained sections.

CO-LOCALISATION CONTROLS

(Exemplified by double-labelling with Anti-X IgG1 and Anti-Y IgG2a)

I. Positive signal only in the appropriate channel
Labelled cells should only be observed in the corresponding channel (and not in other channels)

Slide 1 MAb Anti-X IgG1 -> Goat Anti-Mo-IgG1* Alexa 488 -> Signal observed only in the green channel.
Slide 2 MAb Anti-Y IgG2a-> Goat Anti-Mo-IgG2a* Alexa 594 -> Signal observed only in the red channel.

II. Primary antibody does not react with non-correponding secondary antibody
No signal should be observed (blank sections)

Slide 4 MAb Anti-X IgG1 -> Goat Anti-Mo-IgG2a* Alexa 594 -> No signal
Slide 5 MAb Anti-Y IgG2a -> Goat Anti-Mo-IgG1* Alexa 488 -> No signal

III. Control of secondary antibody specificity
Secondary antibody should only react with proper primary antibody. If staining is observed in non-corres-ponding channel, secondary antibodies interact with each other.

Slide 6 MAb Anti-X IgG1 -> Goat Anti-Mo-IgG1* Alexa 488 + Goat Anti-Mo-IgG2a* Alexa 594 -> Only green signal should be observed

Slide 7 MAb Anti-Y IgG2a -> Goat Anti-Mo-IgG1* Alexa 488 + Goat Anti-Mo-IgG2a* Alexa 594 -> Only red signal should be observed

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