Multi-target immunofluorescence using spectral FLIM-FRET for separation of undesirable antibody cross-labelling

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Indirect labelling techniques of cellular molecules with primary and secondary antibodies for immunofluorescence-based imaging is still serving well for the needs of biologists in advancing the understanding of biological processes. However, these techniques impose a stringent selection of primary and secondary antibody pairs to avoid false-positive immunolabeling leading to misinterpretation, in particular for (co-)localization/interaction of target molecules. At best, primary antibodies raised in different species are used and combined with corresponding secondary antibodies originating in another species or, at least, different to the origin of primary antibodies. Due to this, indirect immunofluorescence is either regularly limited to two - four antigens or a particular combination of target molecule labelling can't be carried out at all.

Here, we present a new method to use this ostensible disadvantage of cross-labelling secondary antibodies by separation of the fluorescence signals by spectral FLIM-FRET. This becomes possible since the undesirable cross-labelling among secondary antibodies leads to the generation of new characteristic FRET emission spectra including a change of the donor lifetime. To demonstrate this, we adapted a sequential labelling protocol and selected appropriate fluorophore pairs on interacting secondary antibodies (e.g. Alexa488 and Alexa546) to achieve strong FRET effects.

As a model, we labelled the target molecules pan-Cytokeratin (Alexa546-rabbit-anti-mouse), TOM20 (Alexa488-goat-anti-rabbit), and golgin (Alexa546-rabbit-anti-mouse). This led to single labelling of TOM20 with Alexa488-goat-anti-rabbit and golgin with Alexa546-rabbit-anti-mouse as well as cross-labelling of pan-Cytokeratin with Alexa488-goat-anti-rabbit and Alexa546-rabbit-anti-mouse. We used an eight channel spectrally resolved FLIM (sFLIM) detection system and acquired data for all labels excited with two lasers pulsating in interleaved mode. Data analysis was performed using the pattern-matching algorithm[1] taking into account emission spectra as well as nanosecond time-resolved fluorescence decays.

The method enabled us to precisely separate all three target molecules generated by just two fluorophore species due to their cross-linking and resulting FRET interaction. Consequently, spectral FLIM-FRET together with pattern-matching analysis forms an excellent tool for use in indirect immunofluorescence by overcoming the undesirable effect of secondary antibody cross-labelling by assigning separate colour channel to cross-linked fluorophores.

1. Niehörster, T. et al. Multi-target spectrally resolved fluorescence lifetime imaging microscopy, Nature Methods, 257-262, 13(3), 2016