Precision localization of cellular proteins with fluorescent Fab-based probes

Vincenzo Manuel Marzullo[1], *Federica* Liccardo[2], *Matteo* Lo Monte[1], *Giuseppe* Palumbo[3], *Marko* Lampe[4], *Giuseppe* Coppola[5], *Alberto* Luini[1]

1. Istituto di Endocrinologia ed Oncologia Sperimentale, Consiglio Nazionale delle Ricerche, Napoli, Italia;
2. Cardiovascular Research Institute, University of California San Francisco, San Francisco, USA
3. Department of Biotechnology and Molecular Medicine (MMBM) School of Medicine, University Federico II, Napoli, Italia;
4. Advanced Light Microscopy Facility, European Molecular Biology Laboratory, Heidelberg, Germany
5. Istituto di Scienze Applicate e Sistemi Intelligenti, Consiglio Nazionale delle Ricerche, Napoli, Italia

Abstract. With continuously improving resolution of today's (super-resolution) microscopes, a major technical limitation of light microscopy based image analysis is linkage error – a visualization error that is measured by the distance between the cellular target to be detected and the fluorescence emitter used for detection. The linkage error of standard labelled antibodies is caused by the size of the antibody and the random distribution of fluorescent emitters on the antibody surface. In this study, we describe a class of staining reagents that effectively reduce the linkage error by more than five-fold when compared to conventional staining techniques. We believe this class of reagents realize an unmet need in cell biological super resolution imaging studies where the precise localization of the target of interest is crucial for the understanding of complex biological phenomena

1 Diffraction-unlimited optical microscopy

Far-field fluorescence microscopy is constantly improving in the continuous attempt to resolve the finest details of intracellular architectures by overcoming the Abbe limitation [1]. This limitation, known as the diffraction limit, prevents the resolution of structures smaller than approximately half the wavelength of light and, as a consequence, makes it impossible to resolve, with high precision, cellular nanostructures that are <250 nm in size. To overcome this limit, super resolution microscopy techniques have been developed. The most relevant improvements to super resolution methodologies consist of a combination of single molecule localization methods (SMLMs), like STORM, and the deterministic approach, that have led to an increased resolving power of microscopes up to a very few nanometres [2,3,4,5].

1.1 Resolution limit and linkage error

1.1.1 Spatial resolution in modern optical microscopy

The unprecedented resolving power of modern instruments [6,7,8], combined with recently developed algorithms for image processing [9,10,11] and for the evaluation of the resolution limit [12] have modified the resolution problem in diffraction-unlimited imaging.

Nevertheless, despite the technological advancement of super resolution microscopy, the localization of precise coordinates for a given cellular protein is still a challenging task. This is essentially due to the fact that in all the fluorescence microscopy techniques, the preferred fluorescence emitters are organic molecules conjugated to affinity probes that bind directly to the cellular epitope of interest.

1.1.2 Localization uncertainty in SMLM

The use of fluorescently labelled probes introduces an error in the localization of the object of interest as the actual position of the biological target within a cell differs from the experimentally determined location due to the distance between the fluorescence emitter and the target epitope. The localization uncertainty introduced by the presence of a linker that physically ties the cellular target with the emitters is called linkage error. This represents a major problem in super resolution imaging analyses since an error with an order of magnitude greater than the nanostructures being visualized can introduce distortions in their reconstruction, especially when performing SMLM studies.

1.2 Spatial displacement of fluorescent emission

1.2.1 Linkage error in SMLM

In SMLMs, linkage errors are identified as the space between the fluorescent label and the target protein; thus, large probes and the distributions of the fluorescence emitters on the probes influence the magnitude of the linkage error. Linkage error is of particular concern when utilizing the largest and most extensive class of detection probes used as microscopy reagents – fluorescently labelled immunoglobulins/antibodies. These use of these reagents in super resolution microscopy is characterized by a target localization uncertainty mainly due to two reasons: the size of the antibody (≈12 nm along the longest axis) and the random distribution of emitters on the antibody probe surface due to the uncontrolled chemical conjugation reactions used to synthesize these probes. So far, several studies have shown that indirect (with labelled secondary antibodies) and direct (with labelled primary antibodies) immunofluorescence techniques can produce a fluorescence displacement of the labels from the cellular target of interest by about 40 nm and 20 nm, respectively [13,14]. When this displacement is measured on the surface of a sphere centred on the fluorophore, the experimental location of the target protein is calculated to be at a distance of up to 20 nm away from the actual intracellular location. This would imply an estimated distribution of the labelling in a fluorescence sphere with a radius of 11.4 nm during the use of full-length primary antibodies as probes and 22.8 nm during the use of labelled secondary antibodies. This results in a corresponding fluorescence displacement of 22.8 nm and 45.6 nm respectively, resulting in a mis-localization of intracellular target coordinates (see Figure 1).

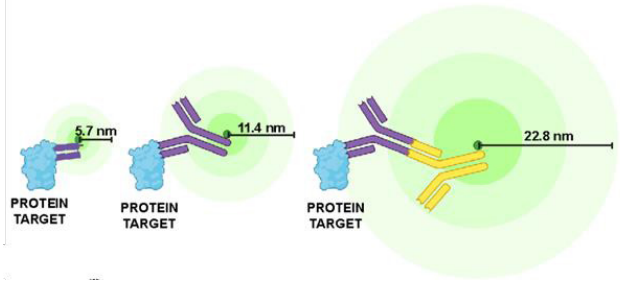

Fig. 1. Pictographic representation of the linkage error.

The most obvious and immediate method for the reduction of linkage error is the development of probes and detection methods alternative to the large full-length antibodies. In sum here we present a promising class of imaging reagents that are easy to produce in the lab with a simple chemical protocol. These reagents reduce the linkage error in single molecule localization microscopy techniques, like STORM, and allow the precision labelling of cellular nanostructures.

References

1. Abbe, E., Anatomie, **9**, 413-468, (1873)
2. Balzarotti, F. et al. Science, **355**, 606-612 (2016)
3. Gwosch, K. C. et al., Nature methods **17**, 217–224 (2020)
4. Gu, L. et al., Nature methods, **16**, 1114-1118, (2019)
5. Cnossen, J. et al., Nature methods, **17**, 59-63, (2020)
6. Wassie, A. T. at al., Nature methods, **16**, 33-41, (2019)
7. Dreier, J. et al., Nature communications, **10**, 556, (2019)
8. Sahl, S. J. et al., Nature reviews, **18**, 685-701, (2017)
9. Ovesny, M. et al., Bioinformatics, **30**, 2389-2390, (2014)
10. Malkusch, S. et al., Scientific reports, **6**, 34486, (2016)
11. Small, A. et al., Nature methods, **11**, 267-279, (2014)
12. Strack, R. et al., Nature methods, **16**, 806, (2019)
13. Früh, S. M. at al., ACS Nano, **15**, 12161−12170 (2021)
14. Aktalay, A. et al, AACS Cent.Sci., **9**, 1581−1590 (2023)