MINFLUX: INTRA-MOLECULAR RESOLUTION AND DIRECT OPTICAL ANALYSIS OF PROTEIN CONFORMATION

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Far-field Optical Nanoscopy

Throughout the 20th century it was widely accepted that a light microscope relying on conventional lenses (far-field optics) cannot discern details that are finer than about half the wavelength of light (>200 nm). However, in the 1990s, it was discovered [1] that overcoming the diffraction barrier is realistic and that fluorescent samples can be resolved down to the nanometer scale. Simple, yet powerful principles allow neutralizing the resolution-limiting role of diffraction. They are the core of STED microscopy and, more generally, have become the basis of far-field optical 'nanoscopy' [2] as an active research field. In brief, fluorophores residing closer than the diffraction barrier are prepared in different molecular states so that they become distinguishable for a brief detection period. Since the process of focusing is no longer utilized for discerning adjacent features, the limiting role of diffraction vanishes, and the interior of (living) cells and tissues can be imaged with diffraction-unlimited resolution using focused light.

A Minimum for Maximum Resolution

The last decade has seen further fundamental progress in the art of optical localization of fluorescence emitters, which has led to imaging resolution on the ~1-3 nm size scale of the studied molecules themselves, and even capabilities to measure *intra*-molecular distances with high precision.

MINFLUX [3-5], a concept for optically localizing photon emitters in space, is at the center of these developments. In MINFLUX imaging the fluorophores are discerned individually by sequential on- and off-switching like in the methods called PALM/STORM. However, whereas in PALM/STORM the localization is based on maximizing the number of detected fluorescence photons on a camera, which is inevitably limited by bleaching, in MINFLUX the molecule is localized by probing it with a deliberately prepared intensity-zero of an excitation beam. For localizing a fluorophore in the focal (x,y) plane, the excitation beam is typically formed as a doughnut The excitation beam is scanned in the proximity of the fluorophore, and the fluorescence is recorded as in a confocal microscope. The position of the molecule is ultimately

identical to the position of the doughnut at which fluorescence emission would be minimal. Importantly, approaching a fluorophore with a position-probing excitation minimum shifts the burden of requiring many photons for localization from the feeble beam of fluorescence photons to the inherently bright and robust beam of molecular excitation provided by a laser. This gives MINFLUX a fundamental edge over popular camera-based localization in terms of photon detection requirements, and hence of speed and precision. The ideas for resolving and tracking molecules with MINFLUX have been successfully integrated into instrumentation that works with a common microscope platform [6] and that is now also commercially available.

The first presentation of MINFLUX [3] had already included examples of tracking fluorescently labeled proteins in cells. However, these movements were random, and the tracking had precisions of the order of tens of nanometers. To more fully realize the strengths and resultant capabilities of the idea behind MINFLUX, and to obtain both high spatial and temporal sampling, an alternative optical implementation of MINFLUX has been based on the interference of pair-wise beams with a defined phase difference, providing line-shaped minima in the x- and the y- direction, replacing the doughnut. Thus, the position of the x-, and y- oriented line-shaped intensity minima can be rapidly scanned by changing the phase difference of the individual beams. This MINFLUX nanoscope has recorded protein movements with a spatiotemporal precision of down to 1.7 nanometers per millisecond, requiring the detection of only about 20 photons emitted by the fluorescent molecule per sampling (time) step [7]. The resolving power of this optical arrangement was initially applied to follow the motor protein kinesin-1 and its stepping on microtubules. Using only a single fluorophore for labeling the kinesin-1, the regular 16 nm steps of individual kinesin heads as well as 8 nm substeps were recorded with nanometer/millisecond spatiotemporal resolution. The results showed that ATP is taken up while a single head is bound to the microtubule, but that ATP hydrolysis occurs when both heads are bound. The attained spatiotemporal resolution also revealed a rotation of the head in the initial phase of each step [7]. These findings were obtained using physiological concentrations of ATP, as was hitherto not possible with tiny fluorescence labels.

Intra-molecular resolution: Ångström-precise distances for macromolecular structural readouts

The push to maximize information from fluorescence emitters recently led to our first demonstrations of resolution *within* macromolecules [8]. Concretely, we showed in proof-of-concept experiments that the MINFLUX method can be used to optically measure the three-dimensional distance between two fluorescent molecular markers, each attached to a specific site of a macromolecule – and this with Ångström precision.

Until now, detecting nanometer distances between two proteins, or their subunits, has been the prerogative of the Förster resonance energy transfer (FRET) method. MINFLUX has now also advanced into this resolution range. At distances of less than 5 to 10 nanometers, the fluorescent molecules, which are about 1 nanometer in size, often interact with each other. As a result, they cannot emit their fluorescence light independently of one another – the prerequisite for a reliable distance measurement. Our

experiments (Fig. 1) therefore used specially developed photoactivatable fluorescent molecules, which can be "switched on" one after the other with a small dose of UV light, but do not interact with each other.

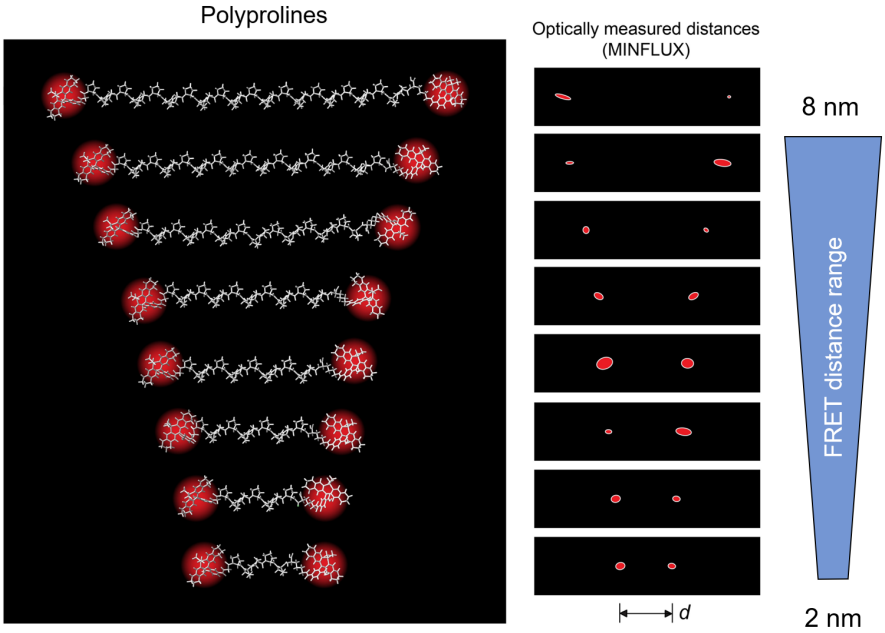

Fig. 1. Intra-molecular resolution with MINFLUX, in the FRET distance range and below. (A) Polyprolines of different lengths, relatively stiff polypeptides, as intramolecular "nanometer rulers" to demonstrate the highest MINFLUX resolutions in the Förster resonance energy transfer (FRET) distance range. The 2-sigma ellipses show the measurement uncertainty of the individual positions. Data from Sahl et al. Science 2024.

In this way, the positions to be measured in the molecule, such as a polypeptide or a protein, could be marked with a single fluorescent molecule and recorded independently. The data in various molecular systems (Figs. 2 and 3) show that with MINFLUX all distances – right down to the direct contact of the fluorescent molecules – can be measured with Ångström precision. To do this, it is sufficient to determine the positions of the molecules in two or three dimensions. The experiments reach the distance range of FRET and even go beyond it. FRET, on the other hand, estimates the distance between two dye molecules indirectly via the energy transfer from one dye to the other. Not only the distance but also the orientations of the dye molecules affect the measurement result. This can lead to uncertainties when it comes to precisely measuring the intra-molecular distance. The FRET method has also often been limited in studies of protein subunits when they move near the limits or outside the measurable distance range (~2-8 nm). The MINFLUX method correctly represents all conceivable distances down to 1 nanometer without any gaps.

To demonstrate precise distance measurement and accuracy, we used a molecule with which the FRET method actually started off in a classic experiment in the 1960s [9]. At that time, Lubert Stryer and Richard Haugland managed to confirm the distance dependence published by Theodor Förster in 1948 [10]. To do this, they used molecular

"rulers" of defined average length called polyprolines (Fig. 1). We employed exactly these rulers in our experiments [8] – and have shown that the method can also be used in cells: fluorescently labeled lamin proteins in human cells, which form approximately 3-nanometer-thin filaments on the membrane around the cell nucleus, were imaged separately using the MINFLUX method. In addition, we demonstrated the potential of the approach through experiments with other small proteins, termed nanobodies, and their oligomers (Fig. 2 a-e)). Using antibody molecules as an example, we showed how multiple position measurements make it possible to resolve the spatial position of the protein subunits relative to one another (Fig. 2 f,g). Using two identical subunits of the bacterial citrate sensor, we demonstrated that even distances of 1 nanometer can be measured. MINFLUX microscopy also clearly revealed the two structural arrangements of the subunits with a precision in the range of 1 Ångström (Fig. 3).

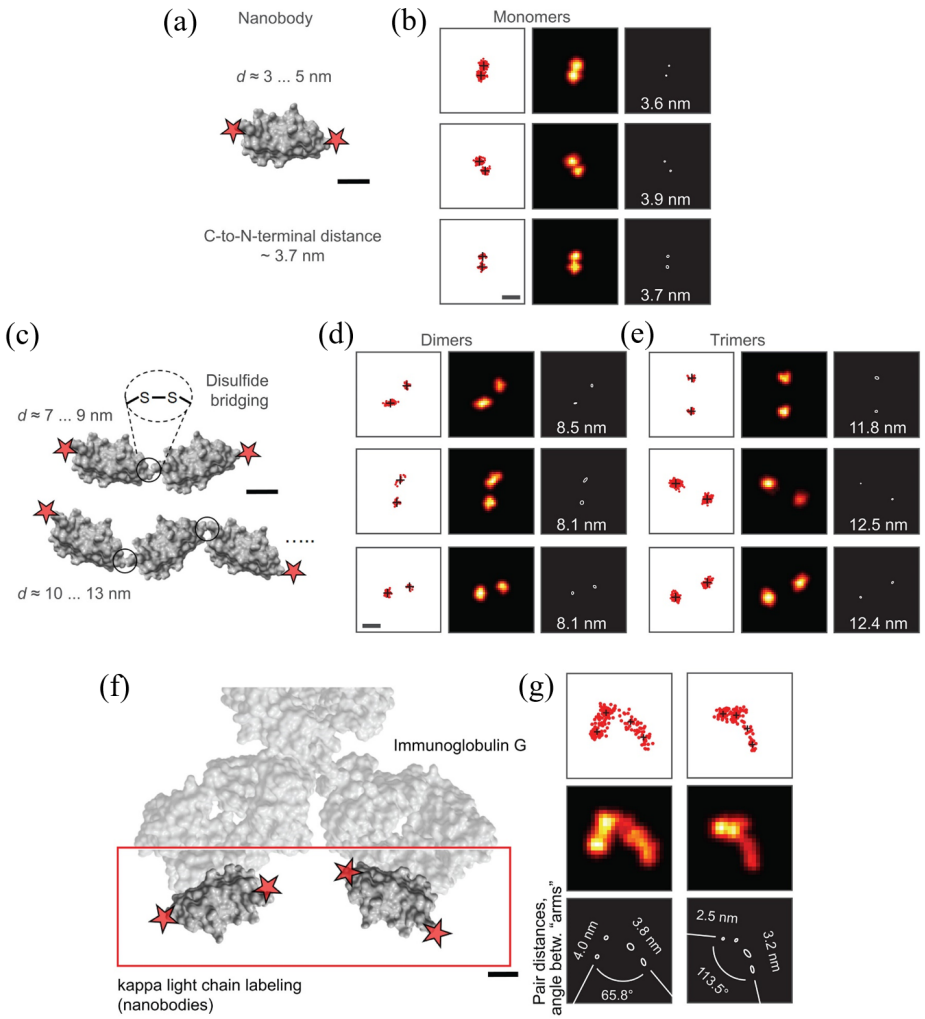

Fig. 2. Intra-macromolecular distance measurements: Sites on proteins and positioning of protein subunits. (a) Camelid nanobody (~16 kDa) with N and C termini cysteine-labeled with a photoactivatable dye. The dimensions of the prolate-shaped nanobody along the longer and shorter axes are ~4 nm by ~3 nm, and its N and C termini are 3.7 nm apart based on crystallographic data. (b) Corresponding MINFLUX example data. (c) Distances attributed to dimers and trimers and measurements corresponding to (d) dimers and (e) trimers. (f) IgG molecule, with the kappa lights chains decorated by N- and C-terminal dye-labeled nanobodies, thus providing up to four positional fluorophore marks (indicated by stars) on the antibody molecule. (g) Examples of different orientations of the two IgG arms. Scale bars: 1 nm [(a), (c), (f)] and 5 nm [(b), (d)]. Data from Sahl et al. Science 2024.

Outlook to future developments

MINFLUX is established as a powerful tool in the repertoire of structural biology for investigating proteins and other biomolecules and their interactions. It is expected that the resulting intra-molecular capabilities will significantly impact the field of protein conformational analysis under near-native conditions. Preliminary data indicate that the protocols can be used to assay e.g. conformational equilibria and changes in the <5 nm range in a fairly routine manner. As a general comment, we remark that being able to resolve *within* macromolecules was not really foreseeable in 2014 at the time the Nobel Prize was awarded. The now thriving field of superresolved fluorescence imaging indeed has come a very long way since.

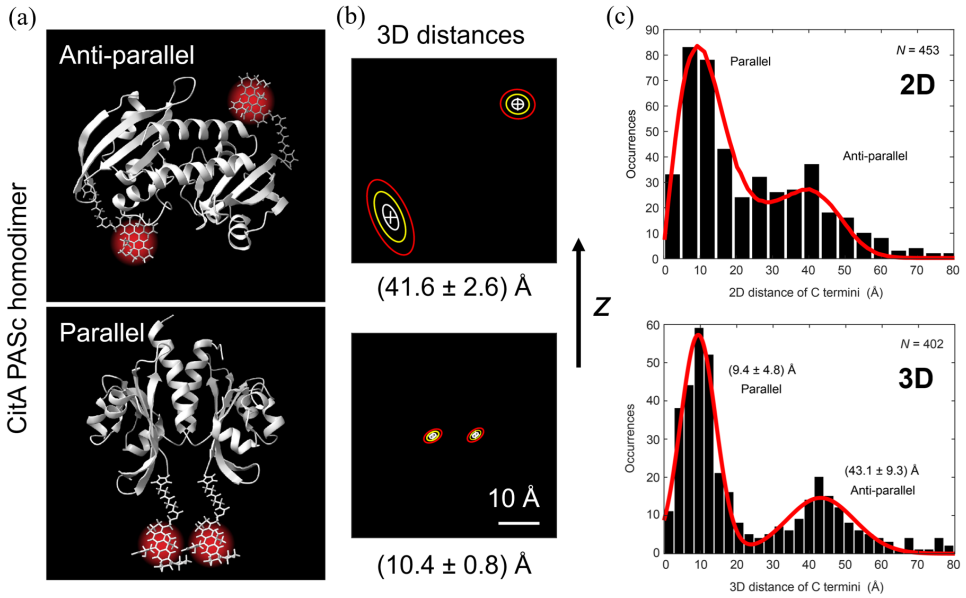

Fig. 3. Direct conformational analysis with MINFLUX. (a) Two identical subunits of the bacterial citrate sensor in antiparallel (top) and parallel (bottom) arrangement. (b) Optical 3D position measurements detect the two states of the dimer formed by the subunits, by very precisely measuring the distances between the fluorescent molecules at the protein ends (C-termini). The ellipses show the measurement uncertainty of the two positions (1, 2 and 3 sigma). (c) Independently measured distributions of 2D (projected) and 3D distances. The set of 3D distances allows to clearly assign parallel and antiparallel dimer measured at (9.4 ± 4.8) Å and (43.1 ± 9.3) Å, respectively. Data from Sahl et al. Science 2024.

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Disclosure
S.W.H. is co-founder and stakeholder in Abberior Instruments GmbH and Abberior GmbH, companies disseminating instrumentation and labeling reagents for super-resolution fluorescence microscopy.

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