Single-Molecule Tracking Approaches to Protein Synthesis Kinetics in Living Cells

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ABSTRACT: Decades of traditional biochemistry, structural approaches, and, more recently, single-molecule-based in vitro techniques have provided us with an astonishingly detailed understanding of the molecular mechanism of ribosome-catalyzed protein synthesis. However, in order to understand these details in the context of cell physiology and population biology, new techniques to probe the dynamics of molecular processes inside the cell are needed. Recent years’ development in super-resolved fluorescence microscopy has revolutionized imaging of intracellular processes, and we now have the possibility to directly peek into the microcosm of biomolecules in their native environment. In this Perspective, we discuss how these methods are currently being applied and further developed to study the kinetics of protein synthesis directly inside living cells.

The protein synthesis machinery translates genetic information into functional proteins. Decades of biochemical research on reconstituted systems have helped paint a detailed picture of this universal, but notoriously complex, mechanism and its main character, the ribosome. (See, for example, ref 1 for a recent review.) From the last 20 years, we can also relate the functional aspects of ribosome-catalyzed peptide bond formation with structures at atomic resolution.2 The core functions of ribosomes are highly conserved throughout the tree of life, where the major differences between prokaryotic and eukaryotic mRNA translation lie in the highly regulated steps of translation initiation, i.e., when the small and large ribosomal subunits assemble on the start codon of the mRNA to initiate polypeptide synthesis. The elongation cycle and the termination events, i.e., when amino acids are connected depending on the mRNA codon sequence and finally released as a polypeptide, are universal both when it comes to codon assignments as well as the catalyzed chemical steps of peptidyl transfer and peptide release.3 Nevertheless, small differences exist between the protein machinery of, for example, bacteria and humans, which we rely on heavily during treatment with antibiotic drugs: many of the clinically relevant antibacterial drugs are targeting the bacterial protein synthesis system.4

Despite this extensive knowledge, there are still missing pieces in our understanding of protein synthesis. In particular, we have a very limited understanding of the dynamic interplay between the protein synthesis machinery and other intracellular pathways, and we still lack quantitative models to connect the molecular details to cell physiology and population biology. For example, why is treatment with the ribosome binding aminoglycoside drug gentamicin bactericidal to a wide range of aerobic Gram-negative bacteria,5 while most protein synthesis inhibiting drugs are only bacteriostatic?6 We know that gentamicin binds the decoding site of the small ribosomal subunit,7 induces misreading of tRNAs,8 and inhibits the translocation step.9,10 But why is this lethal to the cell, while the efficient inhibition of aa-tRNA binding to the ribosomal A site by the tetracycline drugs is only bacteriostatic?11 Clearly, there are big gaps in our understanding of the protein synthesis machinery in its context, and new methods are needed, which allow us to study the process at high enough spatial and temporal resolution directly inside living cells. In this Perspective, we will discuss how recent developments in live-cell single-molecule tracking can provide this missing key. We start off by highlighting the main problems associated with applying these new techniques to protein synthesis. We then briefly review the few different experimental methods that have been used for this purpose so far, and finally, we glance at emerging techniques and discuss the potential of these. Most of the kinetics data on protein synthesis has been retrieved from E. coli, or with purified components from E. coli,1,12 and for this reason, our Perspective is focused on bacterial translation. As mentioned previously, however, many aspects of protein synthesis are universal, and important findings in E. coli are therefore likely to be quite general.

In Vivo Single-Molecule Studies of Protein Synthesis. In any single-molecule tracking approach, there are a few general issues to consider. The technique is highly interdisciplinary and requires adequate skills in three key areas: biochemistry for labeling of the molecule of interest, optics/microscopy for data acquisition at high spatial and temporal resolution, and novel experimental methods that have been used for this purpose so far, and finally, we glance at emerging techniques and discuss the potential of these. Most of the kinetics data on protein synthesis has been retrieved from E. coli, or with purified components from E. coli, and for this reason, our Perspective is focused on bacterial translation. As mentioned previously, however, many aspects of protein synthesis are universal, and important findings in E. coli are therefore likely to be quite general.

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temporal resolution, and data processing for image analysis and mathematical interpretation of the tracking data. The two latter topics are general to most single-molecule tracking experiments, and we will therefore not discuss these in detail. For reviews about the detection methods, we suggest, for example, refs 13 16, and regarding data analysis, we refer to refs 15 and 17 19. Hence, the primary focus of this article will be labeling approaches for studies of protein synthesis kinetics in vivo.

For any fluorescent labeling, a major concern is the attachment of the fluorophore to the molecule of interest and whether this affects the biological function of the molecule. Fluorophores also have different photophysical properties, such as brightness and stability. Whereas imaging and localization can be done in principle with a single frame exposure, direct kinetics measurements require fluorophores that are stable enough to allow for multiple rounds of exposure. For fluorescent labeling in vivo, most studies exploit the convenience of genetically encoded fluorescent proteins (FPs), fused to the protein of interest. FPs are, however, not very photostable, which complicates in vivo kinetics studies.25 Further, in the protein synthesis machinery, many of the key components consist of RNA rather than proteins, and the selectivity of the reactions rely on RNA–RNA interactions. In vivo RNA labeling is much less explored and developed than protein labeling, which is one likely reason why we have seen few single-molecule tracking approaches to protein synthesis studies.

**Fluorescent Fusion Proteins: The Traditional Approach.** Despite moderate brightness and photostability, FPs have been extremely useful for in vivo imaging. Significant bioengineering efforts have improved their photophysical properties, which has resulted in the production of a wide spectrum of FPs allowing multicolor imaging.21 The development of photoactivatable and photoswitchable FPs has allowed for super-resolution imaging (i.e., PALM/STORM, RESOLFT microscopy, reviewed in ref 22) and has simplified single-molecule tracking considerably.23 When the cell size is close to the light diffraction limit, as is the case for many bacterial cells, fluorescence background from off-focus molecules as well as foci consisting of multiple fluorophores drastically complicate single-molecule detection of highly expressed proteins. In this case the use of photoactivatable FPs allow observations of a controlled number of molecules at any given moment. Moreover, stochastic photoactivation of a small portion of FPs can be repeated several times, greatly increasing the number of trajectories obtained from a single cell.23

With respect to the protein synthesis machinery, fluorescent fusion proteins have been used mainly for tracking of ribosomal proteins in different organisms.24,25 In *E. coli*, this approach allowed for determination of the fraction of free ribosomal subunits and their spatial distribution under different growth conditions and antibiotic treatments.26 28 For example, it was found that free ribosomal subunits diffuse throughout the whole cell, whereas elongating ribosomes are nucleoid excluded. Due to the limited photostability of the FPs, however, kinetic studies of subunit association/dissociation have not yet been possible.

Despite the fact that there are a number of crucial protein factors assisting in ribosome-catalyzed protein synthesis, surprisingly few published studies using FP fusions to translation factors exist. In an early single-molecule tracking study, a FP fusion to the stringent response factor RelA was used to indirectly deduce the kinetic mechanism.29 However, the result has been questioned by more recent studies,30,31 and analysis of the RelA–ribosome interactions revealed by cryo-EM structures do suggest a steric clash of the FP in the used construct with ribosome-bound tRNA.32 33 This example highlights another major problem with FPs: they are comparably large. Therefore, rigorous in vivo and in vitro assays, for both the specific function studied as well as for any potential new discoveries, are needed in order to rule out that the FP is compromising the function of the molecule of interest. Considering that translation factors often have several binding partners, and often interact inside the ribosome, the size of FPs might explain why successful labeling in this case has been rare.

Recently, however, Mustafi et al.34 presented an *E. coli* strain, in which both chromosomal EF-Tu genes, i.e., *tufA* and *tufB*, had been modified to encode a C-terminal fusion of the photoactivatable FP mEos2 to EF-Tu. Single-molecule tracking of the fluorescent EF-Tu versions at 2 ms/frame suggested a surprisingly high fraction, 50 60%, of EF-Tu bound to ribosomes. Comparing this number to estimated numbers of ribosomes, the authors concluded that 4 copies of EF-Tu were present on each translating ribosome at any given moment. Whereas the textbook version of translation elongation shows a maximum of one EF-Tu per elongating ribosome, other models have been proposed over the years. Some early in vitro experiments suggested two EF-Tu per aa-tRNA.34 More recent results suggest that the L7/L12 stalk, consisting in *E. coli* of four flexible copies of the L7/L12 ribosomal protein protruding from the large ribosomal subunit, serves as an antenna, channeling aa-tRNA-EF-Tu-GTP ternary complexes toward the A site, irrespective of codon–anticodon match.35,36

The antenna model is also proposed by Mustafi et al. as a possible mechanism to explain their finding. The average dwell time for EF-Tu on ribosomes was estimated to be less than 1 2 ms, which is in line with previous in vitro results suggesting the EF-Tu-dependent steps on elongating ribosomes to require 2.5 ms for productive events (ref 12 at 30 °C as in Mustafi et al.) and, hence, much shorter for nonproductive events. However, as discussed by the authors, since the dwell time of EF-Tu on the ribosome is then likely shorter or comparable to the camera exposure time (2 ms), it was impossible to directly observe pure ribosome bound and free EF-Tu states and transitions between them. Hence, to settle the question regarding the initial interaction of the ternary complex with the ribosome, more sophisticated microscopy techniques with a higher temporal resolution are needed.

Utilizing the same approach as with EF-Tu,37 Weisshaar and co-workers also tracked an FP fusion version of EF-P.37 EF-P (and its archaeal and eukaryotic homologues a/eIF5A) is a universally conserved translation factor, which has recently been shown to assist in translation of poly(Pro) motifs (reviewed in ref 38). By analyzing diffusional step length distributions, the authors estimated the average dwell time of EF-P-mEos2 on ribosomes to 7 ms and that the translation factor binds 25 100% of all elongation cycles. This result suggests that the E site of ribosomes is occupied with EF-P for a significant amount of the time,39 which contradicts the textbook model of three tRNAs per ribosome at any given moment. Stable binding of deacylated tRNA in the E site has, however, been questioned before.40 Analogous to the study by Mustafi et al., the kinetics analysis of the EF-P-mEos2 fusion is hampered by the fact that transitions between ribosome-bound and unbound EF-P were not directly detected. This presents another example of the limitations of conventional camera
peptides, containing an array of SunTag or FLAG epitopes, are correspondingly bound by antibodies between ribosomes as 200 mRNA contained an array of RNA hairpins, which translation in eukaryotic cells. Translation of these specific mRNAs was measured as 1 DNA molecules. The method has previously been used to study mRNA localization and spatiotemporal regulation of translation in eukaryotic cells. Translation of these specific mRNAs could be followed as the encoded polypeptides, consisting of 10–56 tandem repeats of SunTag or FLAG-tag epitopes, specifically bound the corresponding fluorescently labeled antibodies upon emerging from the ribosome nascent peptide exit tunnel (Figure 1). The RNA-binding proteins and peptide-binding antibodies were either labeled by organic dyes in vitro and then microinjected into the cells or expressed in the cells as genetic fusions to FPs. Compared to a simple expression of fluorescent proteins, which due to the maturation time produces a delay between translation and detection, the use of preformed tag-specific fluorophores allowed immediate readout of translation activity. By analyzing changes in intensity of the labeled nascent polypeptides over time, the average translation rate of the defined mRNAs was estimated to be between 3 and 18 amino acids per second, which is in line with previous indirect estimates from genome-wide ribosomal profiling. The initiation frequency on these mRNAs was measured as 1–4 per minute and spacing between ribosomes as 200–900 nt, which is also consistent with previously measured values, obtained using ribosome profiling.

Though the presented studies demonstrate a considerable achievement on direct studies of translation dynamics in living cells, the obtained numbers are averaged over the whole mRNA, and over several ribosomes. In comparison to the ribosome profiling technique, which provides large-scale indirect information about translation activity of endogenous genes with codon resolution, the presented technique has a relatively low throughput. Further, since the mRNA 3’ UTR is sometimes involved in transcription, processing, and degradation regulation of the mRNA, as well as subcellular localization (see ref 52 for a recent review), the introduction of a long array of labeling sites into the mRNA 3’ UTR might cause experimental artifacts. This issue now seems to be solved by the development of the MBSV6 reporter system, with more sparsely distributed RNA hairpins with reduced affinity to MS2 coat proteins, allowing normal mRNA degradation without accumulation of 3’ ends. As with any labeling scheme, careful investigation for possible artifacts, using in this case, e.g., single-molecule FISH (fluorescence in situ hybridization), is however necessary.

**Direct Labeling through RNA Aptamers.** Since there are no known naturally occurring RNA structures with strong intrinsic fluorescence in the visible range (equivalent to the fluorescent proteins), in vivo RNA labeling has been challenging. Fluorophore-binding RNA aptamers have shown great potential, but the labels are not yet bright enough to allow single-fluorophore detection. The technique is based on fluorogenic RNA structures that specifically bind small cell-permeable fluorescent dyes whose intensity increases upon binding (see refs 56 and 57 for recent reviews). In relation to protein synthesis, the so-called Spinach aptamer has been used to specifically label tRNA^Tyr in live E. coli cells. Although the overlap of the intracellular location of the tRNA-aptamer fusion with the spatial distribution of translating ribosomes suggested that the fusion might be functional, the tRNA-aptamer showed a considerably slower activity compared to wt tRNA^Tyr in a reconstituted protein synthesis system.

Even if the fluorescence intensity upon binding is currently too low compared to the background fluorescence from free dyes to allow for single-fluorophore tracking, both structure-guided engineering of the RNA aptamers themselves, as well as exploration of the chemical space for improved ligands, are constantly resulting in improvements of brightness and binding affinity of the complexes. For example, in a recent study, a newly developed member of the Mango aptamer family was used for imaging of noncoding RNAs in live mammalian cells. The aptamer–ligand pair provides improved brightness and photostability as well as a significantly lower dissociation constant compared to, e.g., Spinach, which allowed detection as a basis for tracking. Nevertheless, both studies provide new and interesting insights into protein synthesis and demonstrate the potential of live-cell kinetics measurements.

**Indirect Labeling of RNAs using RNA Binding Proteins.** Whereas FP fusions to ribosomal proteins can be used to distinguish, e.g., a free subunit from one bound to an mRNA, bleaching and blinking of the fluorophores limit the trajectory length, which prevent direct observations of translation events. One way of circumventing this problem is to use an array of FPs. Recently, an approach to follow translation kinetics on single mRNAs inside living eukaryotic cells was developed in several laboratories independently. The approach, which allowed continuous, real-time imaging of translation activity on individual mRNAs, was based on a two-color labeling scheme. The 3’ untranslated region (UTR) of the mRNA contained an array of RNA hairpins, which represent binding sites for several fluorescently labeled MS2 or PP7 bacteriophage coat proteins (originally developed in refs 45 and 46). This allowed long-term localization and tracking of mRNA molecules. The method has previously been used to study mRNA localization and spatiotemporal regulation of translation in eukaryotic cells. Although the overlap of the intracellular location of the tRNA-aptamer fusion with the spatial distribution of translating ribosomes suggested that the fusion might be functional, the tRNA-aptamer showed a considerably slower activity compared to wt tRNA^Tyr in a reconstituted protein synthesis system.
and tracking of bright foci consisting of around ten fluorophores.

**Fluorophores Covalently Attached to RNA.** Site-specific labeling of RNA, in particular tRNA, with small organic dyes has been used extensively for decades in reconstituted protein synthesis systems (e.g., refs 61–64). Compared to FPs, these fluorophores are significantly smaller (0.5–0.7 kDa compared to typically 26–29 kDa), are more photostable (~10^4 photons emitted by a single FP molecule before photobleaching, versus ~10^6 for stable organic dyes65), and particularly outperform FPs in brightness in the red to near-IR range where cell autofluorescence is minimal. For example, the FP mPlm has a brightness (quantum yield times the molar extinction coefficient at absorbance maximum) of 4.1 mM^{-1} cm^{-1},66 whereas the corresponding value for the small organic dye Cy5 is 68 mM^{-1} cm^{-1}.67 A major technical problem for in vivo applications using these small dyes has been to site-specifically connect them to the molecule of interest.

In a series of recent studies, Kapanidis and co-workers have shown that in vitro dye-labeled proteins and nucleic acids can be efficiently delivered into living cells by conventional electroporation68,69 which opens up new possibilities for single-molecule microscopy in vivo. In relation to protein synthesis, Kapanidis’ and Cooperman’s laboratories also electroporated and tracked dye-labeled bulk tRNA in *E. coli* cells.70 While the spatial distribution of the two different diffusional fractions suggested functional binding of the tRNAs to elongating ribosomes, the analysis did not provide any kinetics of these binding events. The main conclusion of the study, that the majority of tRNAs (~70%) are freely diffusing, is further in striking contrast to the suggestion proposed in the EF-Tu tracking study mentioned above, which puts the free tRNA fraction below 1%.33

Shortly after, Volkov et al. utilized the tRNA electroporation method to perform kinetics measurements of protein synthesis in living cells.71 By exploiting the exceptional photostability of the dyes in combination with newly developed Hidden-Markov Model based algorithms for diffusional state transitions analysis72 the authors were able to track Cy5-labeled elongator tRNA^{Phe} and initiator tRNA^{Met} through transitions between ribosome-bound and free states (Figure 2). This study thus provides the first direct measurement of elongation and initiation kinetics with codon resolution in living cells. The resulting tRNA^{Phe} dwell time on ribosomes, 100 ms on average, is perfectly in line with previous in vivo and in vitro results, approximately 50 ms per codon,12,73,74 provided that the deacylated tRNA dissociates quickly from the ribosomal E site.39 The results from tRNA^{Met} tracking suggested a very rapid (20 ms) formation of the elongation competent ribosome once tRNA^{Met} has bound, definitely on the lower limit of in vitro estimated initiation times.5,76

Whereas the kinetics reported in Volkov et al. was limited to ribosome binding events, which distinctly slowed down the tRNA diffusion, the method should in principle allow for analysis of kinetics of the whole tRNA cycle, i.e., including aminoclylation and rebinding to EF-Tu after dissociation from the ribosome. However, with the statistical tools at hand, the exact number of distinct diffusional states could not be reliably determined, as judged from the analysis of simulated tRNA tracking. The number of states also varied to some extent between different strains and conditions. To determine whether these variations can be attributed to the Hidden-Markov Model-based fitting algorithm, or if they reflect actual intra- and/or intercellular heterogeneity in diffusion properties, more focused analyses and tools are needed. In relation to the controversy regarding fraction of free tRNA mentioned earlier, however, these experiments, irrespective of fitting model, suggested that in rapidly growing *E. coli* cells 20–30% of tRNA^{Phe} is freely diffusing at any given moment.

The approach of electroporating in vitro dye-labeled biomolecules for in vivo single-molecule kinetics measurements has a large potential. In vitro single-molecule fluorescence imaging has evolved rapidly during the last two decades. There are several in vitro labeling strategies developed and plenty of already-labeled and assayed components just waiting to be electroporated. Compared to fluorescent fusion proteins, dye-labeled components provide trajectories approximately an order of magnitude longer, which allow accurate tracking and direct observation of molecules during transitions between different binding states within a wide range of diffusion coefficients. There are, however, a couple of issues that have to be considered. First, whereas a properly constructed fusion protein, expressed from the chromosome in a strain lacking the endogenous variant gene, is in most cases functional if the cells survive and grow normally, there is no such guarantee when electroporating the fluorescent components. Hence, rigorous tests are needed, both in vitro and in vivo, to ensure that the diffusing fluorescent particles are functional and actually involved in their normal activities inside the cell. Second, the electroporation approach as presented so far56,71 yields much less data compared to the use of genetically encoded fluorophores. Once the fluorophores are bleached, there is no recovery. In small cells, such as *E. coli*, spatial constraints make it impossible to track several fast-moving molecules at the same time, which means that, in principle, only a single molecule can be tracked per cell. Therefore, at the moment, cell-to-cell comparisons are impossible due to limited statistics. The use of photoactivatable dyes77 will potentially open up for better tracking possibilities also in this case. In eukaryotic cells, many fluorophores can be tracked at once, and the need for photoactivatable dyes is therefore not as obvious. With respect to protein synthesis though, reconstituted eukaryotic systems are still less developed, and the essential in vitro bench-marking of dye-labeled components might therefore present the bottleneck in this case.

Finally, in the study by Volkov et al, the kinetic data was obtained using wild-type tRNA and thus represent averaged numbers for elongation on all Phe codons and initiation on the whole mRNA pool. For studies of sequence- and context-
dependent effects, experimental designs, which connect the kinetics measurements with a particular mRNA or codon, will be needed.

**Outlook.** The protein synthesis machinery provides an excellent example of a complex system where in vitro biochemistry has provided astonishing detail but where a holistic understanding is hampered by insufficient methods for in vivo measurements. Most components are essential, they are abundant (e.g., tens of thousands of ribosomes per E. coli cell, and hundreds to thousands of each tRNA isoacceptor), and they usually operate in asynchrony, which is why experiments in bulk are difficult. Hence, protein synthesis should be the perfect target for live-cell single-molecule fluorescence studies. So far, however, very few reports on the matter exist, possibly due to the fact that the process is mainly based on RNA and due to intrinsic problems with fluorescent proteins, namely, that they are perhaps too big to allow for nondisruptive labeling of most of the protein factors that take part. The use of RNA or proteins labeled with small organic dyes, delivered to the cell through electroporation or any other way, will most certainly open up completely new possibilities. With so many components of the translation apparatus already labeled and benchmarked in vitro, we can expect many new insights once the studies of these molecules are repeated inside living cells. However, whereas in vitro single-molecule fluorescence studies of protein synthesis have mostly relied on FRET or colocalization to follow dynamics of inter- or intramolecular interactions, in vivo single-molecule tracking has so far relied almost exclusively on differences in translational (i.e., location based) diffusion to observe different biochemical states of single molecules. As shown recently, the electroporation approach does open up for intramolecular FRET studies. Inter-molecular FRET, however, such as the pioneering tRNA–tRNA smFRET studies in vitro, will most probably remain a challenge due to the vast number of unlabeled copies present inside the cell and the vanishingly low probability of two differently labeled tRNA species ending up on the same ribosome at the same time. Studies based solely on colocalization are for the same reason also difficult in bacterial cells, and further aggravated by the fact that the localization precision, despite super-resolution, is still not enough to separate single ribosomes in the crowded intracellular environment.

Further, as evident in the discussed EF-Tu tracking study, reactions and interactions during protein synthesis are sometimes very fast, and standard camera-based single-molecule tracking might therefore not be sufficient to resolve the detailed kinetics even with bright long-lived organic dyes. One might expect both cameras and dyes to be improved in the future, but at some point the photon budget of the fluorophore will put a limit on the time resolution that can be achieved using conventional techniques if the spatial resolution is to be kept. There are, however, a number of new technological approaches emerging, with which the time resolution can be improved significantly.

An emerging nanoscopy and tracking method, MINFLUX, allows unprecedented localization precision and recording speed. Compared to PALM/STORM super-resolution microscopy where wide-field illuminated fluorophores are localized through calculations of the centroid of the individual diffraction patterns appearing on a camera, MINFLUX is a beam-scanning system with confocal photon detection, where the exciting beam has an intensity gradient, provided by, e.g., a Gaussian or donut-shaped beam. The position of the fluorophore is calculated based on fluorescence intensities obtained with different beam positions (Figure 3A): three positions surrounding the fluorophore (providing the localization precision) plus one for which the donut hole coincides with the estimated fluorophore position (ideally resulting in minimal photon count and ensuring a unique solution for the localization). This technique allows detection of fluorophore positions with about 20 times higher photon efficiency compared to conventional super-resolution microscopy. For example, MINFLUX has demonstrated localization precisions of ~2 nm from 500 photons to detect organic fluorophores 11 nm apart.

In tracking mode (Figure 3B), the donut-shaped beam follows the fluorophore movement, continuously repeating localization cycles and updating the fluorophore position in a feedback loop. Due to this very efficient photon usage, the use of MINFLUX results in much longer trajectories, in terms of number of positions, compared to conventional wide-field microscopy. In the pilot study, mEos2-labeled small ribosomal subunits were tracked with 8 kHz localization frequency (equivalent to 0.125 ms per localization cycle), resulting in typical trajectory lengths of more than hundred positions. In this particular example, the temporal resolution and the trajectory length outperform conventional camera-based localization techniques, using the same fluorophore, with approximately 1 order of magnitude. The trajectory length was here primarily limited by the blinking of mEos2 and background cell autofluorescence. Most certainly, MINFLUX tracking microscopy will contribute significantly in studies of millisecond and submillisecond time scale dynamic processes in live cells.

All tracking experiments discussed so far rely on detection of the translational diffusion coefficient of the molecule of interest, and changes in this coefficient when the molecule

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**Figure 3. Principle of MINFLUX microscopy.** (A) In static mode, an excitation donut-shaped laser beam performs localization cycles being sequentially placed in three positions around (shown as colored circles) and one closely centered on the expected position of the fluorophore (shown as a red star). The corresponding photon count, registered for each beam position (shown in the bottom), is used to extract the location of the fluorophore. (B) In tracking mode, the beam follows the movement of the fluorophore in real-time, repeating localization cycles around the expected fluorophore position, iteratively updating the position.
bonds to other macromolecules. However, when the time resolution increases, the position displacement between two consecutive observations becomes comparable or less than the localization precision, and another registration signal is needed for reliable detection of the binding state. The detection of rotational diffusion by polarization microscopy or correlation spectroscopy might provide this additional signal. In this case, the fluorescent label needs to be bifunctionally attached to the molecule of interest, so that the orientation of the fluorophore also reflects the orientation of the molecule of interest. The technique further requires a microscope setup with high enough temporal and spatial resolution, as well as single photon detection. Recently, a combination of the MINFLUX tracking principle with correlation spectroscopy and a polarization-sensitive detection scheme was used to experimentally resolve the rotational sliding of a DNA-binding protein along the DNA, for the first time. The study was performed on flow-stretched DNA in vitro, but with the combination of, e.g., electroporation, the same principle should be applicable for detecting changes in binding state also in live cells. In this case, the improved time resolution, down to a sub millisecond level, would provide completely new possibilities for binding kinetics measurements in the living cell.

To conclude, combining experience from decades of in vitro biochemical and structural approaches, with new methods for live-cell single-molecule tracking at high spatial and temporal resolution, we now have the tools to perform biochemical experiments directly inside living cells. Although fluorescent fusion proteins will most certainly be useful for intracellular imaging and tracking also in the future, new techniques are emerging which exploit the extraordinary properties of small organic dyes, simplifying kinetics measurements considerably. With respect to protein synthesis, we can expect that previous conclusions based on in vitro studies will be revisited and to some extent revised when tested in the living cell. We will further be able to study context-dependent effects of protein synthesis, allowing us to connect the detailed knowledge of mRNA translation with other intracellular processes. What are the actual consequences of coupled transcription and translation in bacteria, and to what extent is this used for gene regulation? What is the timing of events during cotranslational targeting of nascent peptides toward the translocon in the cytosolic/ER membrane? These are examples of fundamental questions regarding protein synthesis that cannot be answered using reconstituted systems in vitro given the many different components involved and the geometrical constraints set by the cell.

With these new techniques, we will also be able to study the effect of antibiotic drugs targeting the protein synthesis machinery and the resistance mechanisms against them, on the molecular level, in real time. This will provide us with information regarding the actual binding targets of antibiotics and how this binding prevents cell growth or causes cell death, which has proven difficult to comprehend using existing methodology.

Finally, with emerging microscopy/nanoscopy techniques, we will be able to follow dynamic interactions of molecules inside living cells with an even higher temporal resolution. This will hopefully not only provide us with better kinetics measurements of productive reactions but also the possibility to probe unspecific interactions. This relates to a fundamental question in most areas of molecular biology: how do molecules find their correct targets quickly enough in an enormous space of non- or near-correct targets while still maintaining accuracy? With respect to protein synthesis, some key questions here relate to ribosomes finding the correct start site on mRNAs and the fast and accurate selection of correct aa-tRNA from a vast excess pool of noncorrect species.

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