Single-molecule microscopy in zebrafish embryos

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1. INTRODUCTION

In this thesis, research is described aimed at developing tools of single-molecule microscopy (SMM) to image individual proteins and analyze their dynamic behavior inside cells of living zebrafish (*Danio rerio*) embryos. In this study, the zebrafish model is imaged using SMM in order to investigate mobility patterns of proteins that are anchored in the plasma membrane as well as proteins traversing the cell nuclei. This thesis provides novel applications of SMM for studying mobility patterns of individual molecules in an intact vertebrate biological system, and offers insights into the *in vivo* dynamics of the single molecules under investigation.

1.1 THE ORIGINS OF SINGLE-MOLECULE STUDIES

Because of the emphasis placed on molecules as the minimal functional units of biological systems, research at the single-molecule level plays an important role in a range of fields of biological investigations. Over the years, a myriad of scientific experiments in biology has been conducted with a focus laid on the population level. The outcomes of such experiments are established based on the information retrieved from ensemble analyses, which take into consideration the generalized behavior of the studied population (Shashkova and Leake, 2017). For instance, in ensemble studies performed on cell cultures, it is often expected that members of the populations – comprising thousands of cells – will all exhibit a similar type of action in response to a certain treatment or other biochemical stimuli. While producing the mean of all the observations can eliminate potential outliers present in the data, such as cells exhibiting anomalous behavior, the same approach risks losing valuable information rooted in the biological heterogeneity of all cell populations (Leake, 2013; Rubin, 1990).

The ensemble analyses of biochemical properties lead to a loss of information regarding any deviations in measured parameters, and can ultimately result in misinterpretations of the underlying physiological relevance of existing molecular subpopulations. Therefore, studies at the single-molecule level are especially important in the scientific fields where biological diversity is rife, such as biophysics, systems and synthetic biology (Lenn and Leake, 2012). Ultimately, single-molecule examinations are critical to any research

area where enhancement of both the effective spatial and temporal resolution might shed new light on the processes taking place at the molecular level. Modern techniques enable researchers to directly investigate biochemical interactions of the signal transductions dynamics, which facilitates a more detailed understanding of crucial biological processes, such as protein trafficking, localization, and clustering (Kusumi et al., 2012; Lommerse et al., 2004; Shashkova and Leake, 2017; Shashkova et al., 2017)

One of the most important techniques that is employed to examine biological processes at the single-molecule resolution and that is currently undergoing rapid technical development is the SMM in which fluorescence microscopy is utilized (Miller et al., 2018). This form of SMM has transformed into a valuable method used in many biophysical, biochemical and cell biological studies. Fluorescence SMM allows for observations of the real-time behavior of individual biomolecules, allowing researchers to observe their dynamics and other, detailed features. Proteins, having multiple biological functions, have constituted the primary focus for the majority of SMM studies, and previous research on protein activities performed by SMM helped us better understand a variety of biochemical processes, including protein-DNA interactions or kinetics of protein transport (Yokota, 2020). Single-molecule fluorescence microscopy has uncovered many fundamental biological processes that were not studied previously due to the limitations imposed by ensemble averaging, including studies of the bacterial flagellar motor rotation (Sowa et al., 2005), protein folding and translocation (Bryan et al., 2014), signal transduction (Wollman and Leake, 2016), DNA replication and remodeling (Reyes-Lamothe et al., 2010), oxidative phosphorylation (Lenn and Leake, 2016), cell division mechanisms (Biteen et al., 2008), mitochondrial protein dynamics (Kuzmenko et al., 2011), and many others.

1.2 FLUORESCENCE AND FLUOROPHORES USED IN SMM

The phenomenon of fluorescence occurs when a fluorophore, in a form of an atom or an entire molecule, absorbs a photon and subsequently reemits a photon. Upon absorption of a photon, electrons in the fluorophore transit from a ground state to a higher energy state, in a process that takes less than one femtosecond. After the switch to this excited state, the electron returns to the ground state. This return is accompanied by the emission of a photon, which has a smaller level of associated energy, and therefore

the wavelength of the emission light is longer than the wavelength of the excitation light (Shashkova and Leake, 2017). The loss of energy occurs due to vibrations that result from the oscillations of the atomic orbitals based on the difference in charge distributions between the electrons and the nuclei. The shift in wavelength between the excitation and emission light is named after Sir George Stokes, who was the first person to properly describe the physical foundations of fluorescence (Zimmer, 2009).

A class of fluorophores that is widely used in SMM comprises autofluorescent proteins, such as green and yellow fluorescent proteins (GFP and YFP, respectively). GFP was isolated from the jellyfish *Aequorea victoria* and was first described in 1962 (Shimomura et al., 1962). Around 30 years later, the gene encoding GFP was cloned and in 1992 the first studies were performed in which GFP was expressed in living cells (Chalfie et al., 1994; Prasher et al., 1992). Nowadays, GFP cDNA is often fused to a cDNA encoding a protein of interest and the resulting fluorescent fusion protein is expressed in biological models such as cell cultures, microorganisms or transgenic plants or animals, enabling the detection of this protein in these models by fluorescence microscopy. A variety of GFP mutants are available with modified biophysical characteristics, including the S65T mutant with increased GFP photostability and fluorescence output, the F64L mutant with increased folding efficiency at 37°C, and the A206K mutant without self-oligomerization properties (Zacharias et al., 2002). Moreover, numerous GFP color mutants have been designed, such as T203Y for a yellow fluorescent protein (YFP), or Y66W for a cyan (CFP) one (Lippincott-Schwartz and Patterson, 2003). Other autofluorescent proteins, which are differently colored have been isolated, such as a red fluorescent protein (RFP), which was originally found in *Discosoma sp.,* and served afterwards as the template for several derivatives, of which DsRed and mCherry are most commonly used (Miyawaki et al., 2012; Shaner et al., 2004). In addition, many photoconvertible fluorescent proteins, e.g., mEos, mMaple or Dendra, are currently available and can convert between different excitation-emission spectra of emitted light after illumination with lower (mostly UV) wavelengths. Photoconversion can be used for specific applications such as lineage tracing and protein turnover studies (Baker et al., 2010). Nonetheless, despite all the benefits offered by the use of these autofluorescent proteins, these fluorophores are highly unstable and undergo irreversible photobleaching after a certain time spent exposed to excitation light. Photobleaching is caused by the accumulation of free radicals present in the surrounding aqueous solutions that cause chemical damage to the structure

of the fluorescent proteins. Repetitive fluorophore excitation also contributes to the photobleaching events, as the excited fluorophores might not necessarily emit photon energy in the form of fluorescent light, but enter the dark (triplet) state instead. Being trapped in this state, fluorophores are increasingly vulnerable to undergoing a multitude of photochemical reactions, ultimately resulting in an irreversible loss of their fluorescence, a phenomenon known as photobleaching (Donnert et al., 2009).

As an alternative for labeling proteins with autofluorescent proteins, a multitude of approaches has been designed (Filonov et al., 2011; Los et al., 2008; Stagge et al., 2013; Lippincott-Schwartz and Patterson, 2003). Organic fluorescent dyes with superior characteristics can be attached to proteins of interest through linkers, including HALO- and SNAP-tags. In such cases, the DNA encoding the protein of interest is first genetically fused to the protein tag, i.e., HALO- or SNAP-tag, which consists of a DNA repair protein (in the case of SNAP), or a haloalkane dehalogenase enzyme (in the case of HALO). Following that, the biological sample expressing this fusion protein is incubated with a secondary probe, which is able to covalently bind to the protein tag. The secondary probes are fluorescently labelled with organic dyes, such as coumarin, tetramethylrhodamine, Alexa Fluor dyes, and others. Thus, this methodology enables the use of brighter and more photostable fluorophores when compared to the use of traditional fluorescent proteins. Multiple other labelling strategies are currently being developed or are already available. These include quantum dots, which are nanoparticles that are extremely bright and photostable, spontaneously blinking dyes (Bentolila et al., 2005), such as hydroxymethyl Si-rhodamine, HMSiR, which exploit reversible, pH-dependent chemical reactions and enable performing SMM at defined pH values in aqueous solutions (Uno et al., 2014), and temporally blinking dyes, such as PAINT, which do not require fluorophore switching and instead employ dyes that freely diffuse until their interactions with target molecules via permanent or transient binding (Giannone et al., 2010).

1.3 THE DEVELOPMENT OF SINGLE-MOLECULE MICROSCOPY (SMM)

SMM represents a family of powerful imaging techniques with a high spatial resolution that are used to visualize biological structures at the molecular scale. Due to diffraction, an image of a point source of light, visualized using a lens-based microscope, is not a single point but a point spread function (PSF). The PSF is usually an Airy disc pattern

with a central Gaussian peak, being the diffraction maximum, that is surrounded by less intense concentric rings. This PSF results in the blurring of any structures with a size that is smaller than the size of this central peak. This diffraction limit determines the resolution of an optical system and as a result, the capacity of an optical microscope to resolve the subcellular localization of single molecules and complexes thereof. In a practical sense, the resolution of a microscope is defined as the smallest separation distance between two point-like objects at which they can still be distinguished as individual objects. Therefore, the majority of resolution criteria, including the Sparrow limit and the Rayleigh criterion, refer to the properties and the geometry of the PSF (Sheppard, 2017). The Rayleigh criterion, for instance, states that two point sources of light are only resolved when the center of the Gaussian peak of one Airy disc overlaps with the middle of the first dark ring that surrounds the other one, or is further removed from the center of the other disc. Otherwise, the two PSFs merge and the two sources cannot be distinguished. The Rayleigh criterion enables the calculation of the resolution of a given microscopy setup and is described by equation 1:

$$Rayleigh\ Resolution_{x,y} = \frac{0.61\lambda}{NA} \quad (1)$$

where λ denotes the light wavelength, and NA is the numerical aperture of the objective used in the system. Hence, for an optical system using an objective with an NA of 1.49, often employed in SMM studies, the resolution in the visible light spectrum (λ from 380 to 680 nm) ranges between 155 and 278 nm (Chen et al., 2016; Lelek et al., 2021).

In general, SMM techniques are based on the premise that the spatial coordinates of single fluorescent molecules can be identified with high precision by determining the location of the center of their PSFs, if these PSFs never overlap. Using this approach, the localization precision is bound by the signal to noise ratio (SNR), but not by the wavelength of light or the pixel size (Lelek et al., 2021). Microscopy techniques that make use of this localization method most often keep the concentration of active fluorescent molecules low to avoid the overlap between the PSFs of the individual fluorescent molecules. Examples of this approach include super-resolution techniques, such as photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), which are based on low concentrations of fluorescent molecules, resulting from photoactivation or photoswitching of only a subpopulation of fluorophores.

Both PALM and STORM have been extensively used in cancer biology research, including the visualization of exosomes in human breast and cervical cancer cells (Chen et al., 2016; Lelek et al., 2021).

Thus far, many different technical approaches have been applied to perform fluorescence SMM. Imaging at the single-molecule level requires detection of signals from individual fluorophores and hence low background fluorescence levels. This can be achieved through limitations imposed on the illumination volumes, thereby reducing the number of excited fluorophores present in the illuminated sample. The first developed microscopy technique designed to reduce the illumination volume was confocal microscopy, established by Minsky in 1961 (Minsky, 1988). In this technique, a pinhole that is conjugate to the sample plane is placed in front of a detector. The incident light that is focused on the pinholes reaches the detector, whereas the out-of-focus light does not pass through the pinhole and thus does not reach the detector. Consequently, the use of confocal microscopes significantly improves the SNR in the recorded images since it removes out-of-focus fluorescence.

The earliest reports of SMM, which focused on imaging myosin and its ATPase in an aqueous solution, utilized total internal fluorescence (TIRF) microscopy, which dramatically decreases the background signal by reducing the illumination volume (Funatsu et al., 1995). TIRF microscopy is founded on the optical principle of evanescent waves, which are generated when an incident light beam is totally reflected at the interface between two media with a different refractive index. This phenomenon occurs when the first medium has a larger refractive index than the second medium, and when the light rays reach the interface at an angle of incidence larger than the critical angle, which is achieved when the angle of refraction is 90° or larger. In fluorescence microscopy, TIRF occurs when a laser beam arrives at the interface between a coverglass and the aqueous environment of a sample. The critical angle for water and glass media equals approximately 62°, above which, at the interface, a total internal reflection of the laser beam into the coverglass occurs instead of it being transmitted through the water. As a result, an evanescent wave is created just above the coverglass that can be used to excite fluorophores located within 100 to 150 nm above the coverglass. Hence, TIRF is suitable for the detection of single molecules that are associated with the plasma membranes of cells or molecules that are immobilized on glass coverslips (Shashkova and

Leake, 2017; Yokota, 2020). TIRF microscopy has been used as an SMM tool to study, among other biodynamic processes, cytoskeleton assembly mechanisms, microtubule interactions, presynaptic filament dynamics, single lipopolysaccharide transfer onto the toll-like 4 receptors, and capping of individual actin filaments in vertebrate organisms (Kim, 2018; Ross and Dixit, 2010; Stoppin-Mellet et al., 2020, Fujiwara et al., 2016; Kuhn and Pollard, 2007; Umemura et al., 2008)

In other early SMM studies, conformational dynamics of single proteins and other molecules were studied by single-molecule Förster resonance energy transfer (smFRET). This technique employs the principle of energy transfer between fluorescent donor and acceptor molecules. If these molecules are close to each other, i.e., within less than 10 nm from each other, the donor in an excited state relays its energy to an acceptor, which then gets excited and subsequently emits light. FRET is commonly utilized in research focusing on molecular interactions, such as protein-protein and protein-nucleic acid associations, as well as on conformational changes of enzymes and nucleosomes, protein folding, and dynamics of intrinsically disordered proteins (Buning and van Noort, 2010; Koopmans et al., 2007; König et al., 2015; Metskas and Rhoades, 2020; Ha et al., 1999)

1.4 MICROSCOPY SETUPS USED FOR SMM

SMM techniques require a wide-field microscope equipped with standard, continuous-wave lasers for fluorophore excitation together with a camera sensitive enough to detect single molecules. A plethora of both commercial and custom, open-source hardware implementations have been developed. Most of SMM setups utilize scientific or industry grade, powerful lasers with a power of at least 100 mW (Holm et al., 2014; Kwakwa et al., 2016). A dichroic filter with multiple passbands might be selected to reflect excitation laser lines onto the sample and transmit emission wavelengths to the detector. In the case of multicolor microscopy, multiple laser lines can be combined and co-aligned using dichroic mirrors. The fluorescence emission of the specimen is imaged through an objective lens. Typically, 60X to 100X oil immersion objectives with a NA of 1.4 or higher are used to ensure efficient photon collection (Fig. 1A). Most commercial microscopes for SMM contain in-built automated control systems to keep specimens in focus. When imaging molecules located close to the interface of the sample and the coverslip, such as cellular membranes, TIRF microscopy along with its derivative, highly

inclined and laminated optical sheet illumination (HILO), can be used to reduce the out-of-focus background and improve SNR (Fig. 1B). Several methods exist that facilitate SMM imaging in three dimensions, for example by inserting a cylindrical lens into the optical path (Lelek et al., 2021; Mashanov et al., 2003).

For high sensitivity detection of the emitted light, an electron-multiplying charge-coupled device (EM-CCD) or a scientific complementary metal-oxide-semiconductor (sCMOS) camera is generally used. The EM-CCDs enable the detection of single photons with reduced noise levels and are particularly suited for structures with low photon counts. On the other hand, sCMOS cameras are less sensitive to signals with low photon counts, but have higher frame rates that allow imaging larger Field of Views (FoVs), and achieve SNRs similar to EM-CCDs for bright fluorescent dyes. At the detector, the pixel size should be approximately equal to the PSF standard deviation, typically in the range of 100 to 150 nm. The optimal magnification should be determined individually, taking into account specific background and photon numbers, both inherent to the microscopy setup chosen (Thompson et al., 2002; Tokunaga et al., 2008).

1.5 SMM TO STUDY PROTEIN DYNAMICS *IN VIVO*

The applications of SMM have also been extended to study the dynamic behavior of the proteins inside living zebrafish (Schaaf et al., 2009). Zebrafish embryos, due to their optical clarity, are excellent model organisms for visual analyses of biodynamic processes and for research using fluorescently labeled molecules (Canedo and Rocha, 2021; Detrich et al., 2011; Garcia et al., 2016; Gore et al., 2018; Lieschke and Currie, 2007). Furthermore, the high fecundity and short generation time of the model facilitate genetic screens and the identification of mutant phenotypes (Haffter et al., 1996; Reisser et al., 2018). Using TIRF to reduce out-of-focus excitation, the mobility pattern of YFP fused to the membrane anchor of the human H-Ras protein was determined in the apical membranes of cells in the outer epidermal layer of two-day-old zebrafish embryos. This study showed the differences between cultured cells and proper *in vivo* models at the single-molecule level, thereby underlining the importance of applying SMM in intact living organisms.

In the research described in this thesis, we aimed to further extend the possibilities of SMM in zebrafish embryos beyond imaging of proteins in the epidermal cell membranes. Therefore, to enable visualization of proteins in the cytoplasm or nuclei of cells anywhere in the zebrafish embryos, two microscopy approaches have been used that until recently had not been employed as SMM tools before: Light-Sheet Fluorescence Microscopy (LSFM) and Two-Photon Excitation Fluorescence Microscopy (2PEFM).
In LSFM, two objectives, illumination and a detection objective, are positioned orthogonally to each other. A plane of excitation light, called a light sheet, is sent from the illumination objective through the specimen, exciting only the fluorophores present at the detection focal plane. Photons emitted by the fluorophores in the illuminated sample section are collected by the detection objective. This microscopy technique enables fast imaging with a low background signal as well as optical sectioning of specimens. However, the use of LSFM in SMM applications has been limited due to spatial constraints imposed by the geometry of the objectives, meaning that the size of the objectives with high NAs, necessary for the SMM imaging, has not always been compatible with the designs of many LSFM setups. This problem can be circumvented by the use of long-working-distance air objectives for the illumination, or by using LSFM configurations that employ a modified light path, such as prism-based LSFM (PCLSM) and reflected LSFM (RLSFM) (Bernardello et al., 2021). LSFM has been used to visualize fluorescently labelled molecules in aqueous solutions, cell nuclei of salivary glands extracted from *C. tentans*, and nuclei of cultured cells (Gebhardt et al., 2013; Ritter et al., 2010).

In 2PEFM, a fluorophore is simultaneously excited with two photons. The use of two photons means that approximately half the energy per photon is required to excite a molecule, extending the wavelength of the excitation light to the near-infrared (NIR) spectrum. Two-photon excitation (2PE) is achieved by using pulsed laser sources in combination with focusing the laser beam (Benninger and Piston, 2013). The pulsed laser sources generate high peak intensities that are necessary for 2PE, whereas the average laser power remains relatively low, reducing linear absorption, heating, and phototoxic damage to the sample (Soeller and Cannell, 1999). Focusing the laser beam with the objective lens ensures the selective excitation of fluorescent molecules in the confocal volume, which means that the background fluorescence is eliminated without the use of a pinhole. A major benefit of the 2PEFM technique is that biological tissues are exposed to less light scattering and absorption at NIR than at visible wavelengths,

allowing for imaging fluorescent signals located deeper in the tissues of a biological model. The 2PEFM technique has, for instance, been used to perform live imaging of transient neuronal Ca^{2+} fluxes in rodent brains (Schwille et al., 1999; Stosiek et al., 2003).

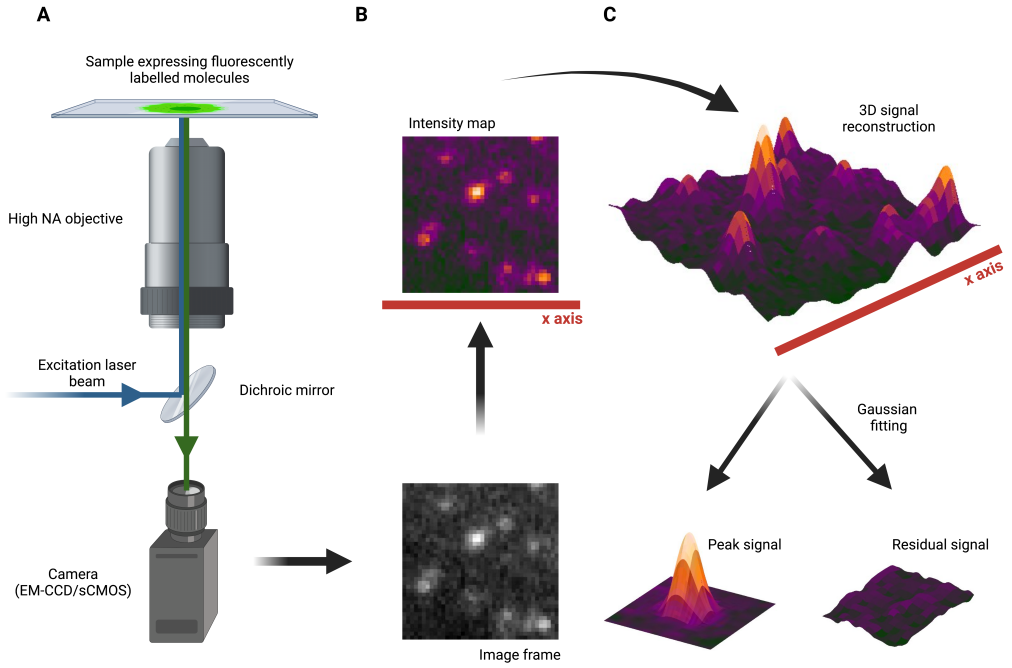

FIGURE 1: Representative image of the SMM setup used to visualize and analyze single fluorescent peaks. (A) A schematic of an SMM setup used to visualize single molecules. Fluorescently labelled molecules present in a sample are excited by an incident laser beam and images are acquired using an objective with a high NA. The collected fluorescence signals pass through the dichroic mirror and are then recorded by a camera. **(B)** An example of an image obtained through SMM (below) and its color-coded version (above), presented to visualize differing intensity levels. **(C)** A 3D signal reconstruction of a frame presented in (B). Based on this 3D fluorescence intensity map, a 3D Gaussian fitting can be performed, and by determining the center of the Gaussian peak the location of the fluorescent molecules in the sample can be determined with high precision.

1.6 ANALYSIS OF THE DATA OBTAINED THROUGH SMM IMAGING

An extensive analysis of the mobility patterns of single molecules requires detailed quantification of the localization and displacements of molecules throughout the imaging process (Fig. 1B). In this doctoral thesis, we employ various methods that have

been designed to describe the dynamic behavior of the investigated populations of molecules. This analysis begins with the localization of the molecules with high positional accuracy, by fitting the detected fluorescence intensity signals to a three-dimensional Gaussian function (Fig. 1C). The peak of the Gaussian function denotes the location of a molecule, whereas the height of this function, which provides information on the intensity, and its full width at half maximum (FWHM) are both used as selection tools to differentiate between single fluorescent molecules and their aggregates. The band-pass filters, in turn, are the tools used to reject signals having unwanted parameters and background noise (Fig. 1C) In order to distinguish different subpopulations of molecules and to establish values of diffusion coefficients and the potential existence of confinement areas, custom-designed, Particle Image Correlation Spectroscopy (PICS) software is used (Semrau and Schmidt, 2007). This software does not track individual particles but determines correlations between the location of molecules in consecutive frames. In this way, cumulative probability distributions of all correlations are plotted, and by subtracting all random correlations, distribution plots of all squared displacements between consecutive frames are generated. By fitting these plots to population-based models, the number of molecular fractions present in the studied populations and their mean squared displacements are determined. Subsequently, this procedure is repeated for different time lags. To evaluate whether any of the populations confine to specific areas, the mean squared displacement values of the populations are plotted against the time lag. These plots are then fitted either to a free Brownian diffusion model, represented by a linear function, in which the molecules move freely and are unconstrained, or a confined diffusion model, represented by an exponential function that reaches a plateau, in which the molecules also move freely, but their movement is confined to a specific area surrounded by impermeable barriers.

It is well established that many proteins fulfil their biological functions not as a monomer but in the form of polymers. It has been suggested that more than 35% of protein molecules in cells are polymeric (Goodsell and Olson, 2000). Single-molecule imaging can be used to quantify the number of biomolecules in a polymer or a cluster, which facilitates understanding the stoichiometry of these complexes (Marianayagam et al., 2004). Single-step photobleaching (SSP) analysis is a straightforward and powerful method that allows for the counting of molecules within a protein complex. The stoichiometry is determined by observing the number of discrete descending steps as an

effect of photobleaching of individual fluorophores comprising the complex. In this doctoral thesis, the SSP analysis is performed using the ImageJ plug-in TrackMate (Tinevez et al., 2017). The detection of individual particles is performed by determining a maximum intensity value in a circular selection with a predetermined diameter, which denotes an individual molecular complex. The TrackMate software also enables trajectory reconstruction of the localized particles and determines, therefore, whether they show the same type of dynamic behavior throughout this trajectory, or rather switch between different molecular subpopulations with different mobility patterns.

1.7 H-RAS AND THE GLUCOCORTICOID RECEPTOR (GR): MODEL PROTEINS FOR SINGLE-MOLECULE STUDIES IN THE MEMBRANE AND THE NUCLEUS

Two model proteins have been used in this doctorate project. For investigations of the protein mobility patterns in the plasma membranes of epidermal cells in the living zebrafish embryos, the human H-Ras proteins have been used (Chapters 2 and 3). For studies focusing on the mobility patterns of nuclear proteins in cells of zebrafish embryo yolk syncytial layer (YSL), the glucocorticoid receptors (GR) have been utilized (Chapter 4).

H-Ras is an important signaling protein that is mainly localized at the plasma membrane in numerous cell types of vertebrate organisms, though some fractions have also been reported to exist in membranes of endosomes, the endoplasmic reticulum and the Golgi apparatus. It is a member of the Ras protein family that activate intracellular signaling cascades and regulate crucial biological processes taking place in various cells, such as growth, proliferation, and differentiation (Malumbres and Barbacid, 2003). Ras proteins are small GTPases that switch between an inactive, GDP-bound state, and an active, GTP-bound state that delivers signals from the tyrosine kinase receptors activated by growth factors at the cell surface, to promote proliferation and survival programs. Upon growth factor binding, the receptors transmit the extracellular signal through its transmembrane domain, resulting in the growth factor activation and phosphorylation of the tyrosine kinase receptor. The phosphorylated receptor recruits the son of sevenless (SOS) protein via several adaptor proteins, such as GRB2. The SOS protein, being a guanine nucleotide exchange factor, leads to the Ras activation by exchanging GDP by GTP. Subsequently, the active, GTP-bound form of Ras binds to the

RAF kinase, promoting its dimerization and activation. Activated RAF phosphorylates and activates MEK, which, in turn, phosphorylates and activates ERK. Finally, activated ERK phosphorylates a number of substrates, including the transcription factors Elk-1, and serum response factors (SRFs), which then trigger transcriptional programs related to cell cycle progression, protein translation, angiogenesis, metastasis, and cell death evasion (Fig. 2A) (Downward, 2003; Samatar and Poulikakos, 2014). Various isoforms of Ras proteins exist, such as H-Ras, K-Ras and N-Ras, which largely differ in their carboxyl-terminal hypervariable region (HVR), formed by 25 amino acids. The most carboxyl-terminal part of the HVR is an anchor that is responsible for attaching Ras proteins to the cytoplasmic leaflet of the cell membranes upon posttranslational lipidations. In H-Ras, the anchoring domain consists of the CAAX motif, which is responsible for signaling the posttranslational, prenyltransferase-mediated addition of farnesyl or geranylgeranyl lipid groups that anchor the H-Ras protein in the cytoplasmic leaflet of the plasma membrane (Brunsveld et al., 2009; Willumsen et al., 1984).

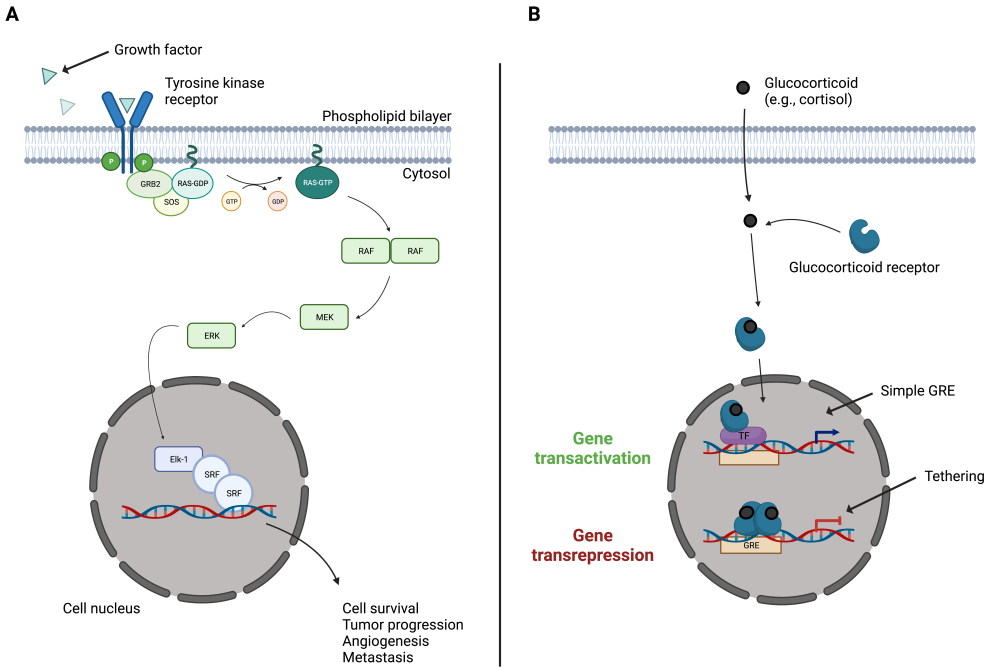

FIGURE 2: Biochemical signaling pathways of the model molecules employed in the research described in this thesis. (A) The H-Ras protein is an early-stage player in the signaling pathway leading to cell cycle control and apoptosis evasion. After activation by a growth factor, a receptor tyrosine kinase

recruits several proteins, which results in Ras activation by exchanging GDP with GTP. Subsequently, in its active, GTP-bound form, Ras activates RAF kinase, what starts the activation cascade leading to the ERK activation. Ultimately, ERK phosphorylates a number of substrates in the cell nucleus, which execute transcriptional programs related to cell cycle progression, protein translation, angiogenesis, metastasis, and cell death evasion **(B)** The GR protein is an important factor that controls many processes ranging from cell metabolism to blood pressure. Upon activation by a glucocorticoid ligand (i.e., cortisol), the GR translocates to the nucleus. In the nucleus, the GR acts as a transcription factor (TF) and regulates the expression of many genes by binding to specific DNA sequences, called glucocorticoid response elements (GREs), and by interacting with other TFs participating in RNA transcription. Legend: GRB2 – growth factor receptor-bound protein 2, SOS – son of sevenless, Elk-1.- transcription activator, SRF – serum response factor.

The GR is a well-studied steroid receptor, which mediates the effects of glucocorticoids, such as the endogenous stress hormone cortisol. Upon activation by cortisol, the biological function of the GR is to maintain metabolism and energy homeostasis. The receptor is also involved in controlling blood pressure through the inhibition of vasodilators, and has been implicated in memory formation as well as the proper functioning of the cells involved in the immune system response. Because of their suppressive effects on the immune system, synthetic glucocorticoids, such as prednisone or dexamethasone, are widely used clinically as anti-inflammatory drugs. Upon activation by a glucocorticoid ligand (i.e., cortisol), the GR translocates to the nucleus, where it acts as a transcription factor (TF). The GR regulates the expression of a large number of genes by binding to specific DNA sequences, called glucocorticoid response elements (GREs), and the stimulation of the expression of GRE-dependent genes, what is generally known as transactivation. Alternatively, the activated GR may also interfere with the activity of other TFs, including nuclear factor kappa B (NF-□B), a mechanism that is commonly referred to as transrepression (Fig. 2B) (Groeneweg et al., 2014; Keizer et al., 2019). Both the human and the zebrafish genomes contain only one gene encoding a GR protein, and it has been shown that the zebrafish GR displays a high level of similarity to its human counterpart at the structural and functional levels (Schaaf et al., 2008; Kadmiel and Cidlowski, 2013; Liu et al., 2019).

1.8 OUTLINE OF THE DOCTORAL THESIS

The aim of the project described in this doctoral thesis is to extend the applications of SMM techniques to study the dynamics of single proteins *in vivo*. The outcomes of the

thesis show that it is possible, using TIRF, LSFM and 2PEFM techniques, to study the mobility patterns of single and fully functional proteins in living zebrafish embryos, both at the plasma membranes as well as in the nuclei of embryonic cells. Furthermore, the results shed new light on the single-molecule dynamics of the model proteins used and offer new perspectives on single-molecule data interpretation. This thesis consists of three experimental chapters:

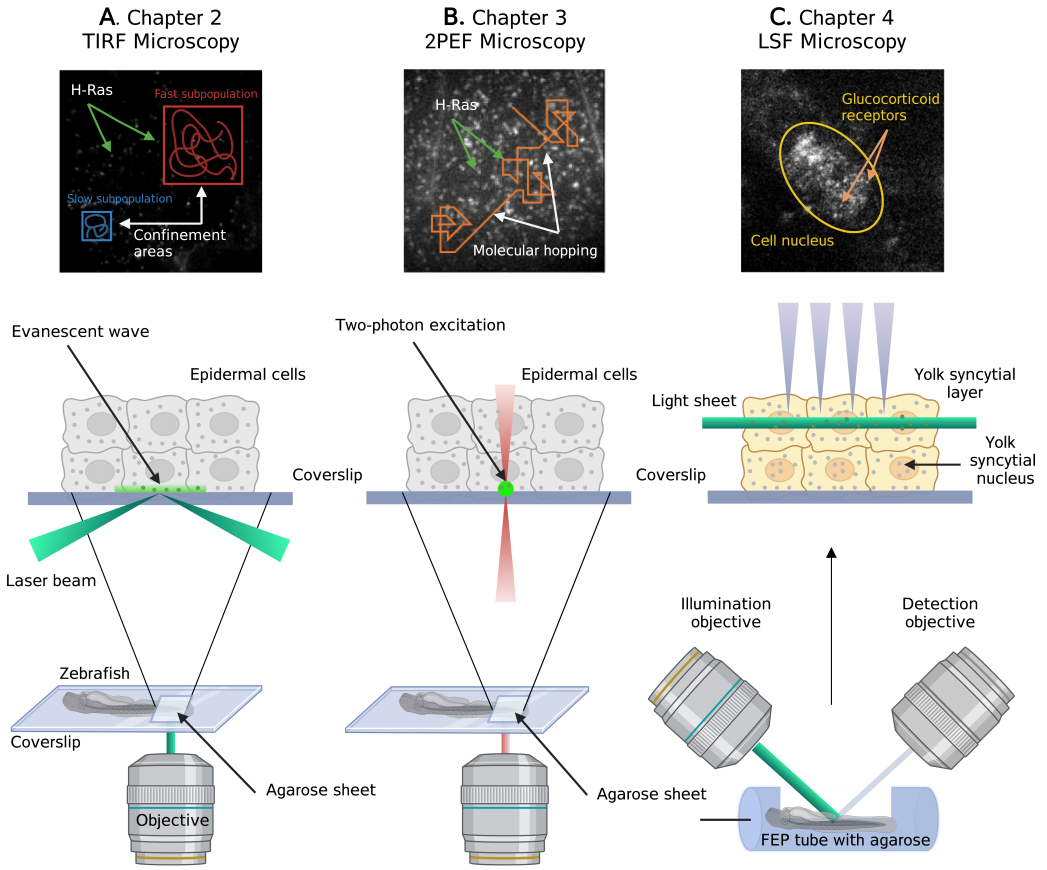

FIGURE 3: Single-Molecule Microscopy (SMM) techniques employed in the research described in this thesis. (A) TIRF microscopy has been used to determine mobility patterns of the full-length H-Ras proteins, its membrane anchors and genetic mutants. In the TIRF microscopy technique, an incident laser beam is positioned against the coverglass-sample interface at the angle larger than the critical angle, creating an evanescent wave that excites fluorophores in the epidermal cell membranes of the zebrafish embryo. Analysis of the TIRF microscopy data allows for differentiation between different protein

subpopulations and determination of their initial diffusion coefficients and the size of their confinement areas. **(B)** 2PEF microscopy dramatically reduces photobleaching and has been used to image H-Ras membrane anchors over a prolonged period of time as well as to reconstruct their molecular trajectories. Fluorophore excitation with two photons takes place only in the focal plane of an image, due to the highest photon density in that area. In the two-photon excitation mode, out-of-focus fluorescence signal is eliminated. This technique enables determination of different dynamic states exhibited by a single H-Ras anchor particle, and visualization of anomalous diffusion patterns, such as 'hop' diffusion. **(C)** LSF microscopy has been used to determine mobility patterns of GR proteins in the yolk syncytial nuclei of zebrafish embryos. In this technique, illumination and detection objectives are separated and positioned orthogonally to each other. The light sheet created by the illumination objective enables excitation of the fluorophores located deeper in the zebrafish embryo tissues and, for instance, studies on the dynamics of transcription factors in the cell nucleus.

In **chapter 2**, SMM is applied *in vivo*, using the zebrafish embryo model. The dynamics of the membrane protein H-Ras is studied using a TIRFM setup and compared with those of its membrane-anchoring domain, C10H-Ras, and several H-Ras mutants (Fig. 3A). The results confirm the presence of a fast- and a slow-diffusing subpopulation of molecules, which both confine to microdomains within the plasma membrane. The constitutively active mutant H-Ras^{V12} exhibits higher diffusion rates and is confined to larger domains than the wild-type H-Ras and its inactive mutant H-Ras^{N17}. Subsequently, it is demonstrated that the structure and composition of the plasma membrane have an imperative role in modulating specific aspects of H-Ras mobility patterns and that differences between individual cells within the epidermis of the embryo largely contribute to the overall data variability

In **chapter 3**, the dynamics of GFP fused to the H-Ras membrane-anchoring domain, C10H-Ras, is studied using a 2PEFM setup. With the two-photon excitation mode, which substantially increases the stability of the fluorescent proteins, it is possible to follow single C10H-Ras particles over a long time and reconstruct their molecular trajectories (Fig. 3B). The trajectories exhibit that the slow-diffusing population of GFP-C10H-Ras fusion proteins show a dynamic behavior that is referred to as 'hop diffusion', and is characterized by long periods of slow diffusion in a confined area that are intermitted by brief periods of relatively fast diffusion, i.e., hops, over longer distances.

In **chapter 4**, an LSFM setup is utilized for imaging individual GRs inside nuclei of YSL cells in living zebrafish embryos over a prolonged time. The optical configuration of the

setup can readily make this SMM approach available in many laboratories, whereas a dedicated sample-mounting system ensures sample viability and mounting flexibility (Fig. 3C). To validate the robustness of the LSFM setup, the GR mobility patterns are analyzed with and without the administration of dexamethasone. As anticipated, dexamethasone administration significantly alters the dynamics of GR. By further characterization of the sources of variation in the *in vivo* results, it is found that most of the variability in the results comes from imaging different areas within an individual zebrafish embryo.

In **chapter 5**, the outcomes of this thesis are summarized and discussed. Future perspectives of the SMM techniques for *in vivo* applications are provided.

REFERENCES

Baker, S. M., Buckheit, R. W. and Falk, M. M. (2010). Green-to-red photoconvertible fluorescent proteins: tracking cell and protein dynamics on standard wide-field mercury arc-based microscopes. *BMC Cell Biol.* **11**, 15.

Benninger, R. K. P. and Piston, D. W. (2013). Two-photon excitation microscopy for the study of living cells and tissues. *Curr. Protoc. Cell Biol.* **Chapter 4**, Unit 4.11.1-24.

Bentolila, L. A., Michalet, X., Pinaud, F. F., Tsay, J. M., Doose, S., Li, J. J., Sundaresan, G., Wu, A. M., Gambhir, S. S. and Weiss, S. (2005). Quantum dots for molecular imaging and cancer medicine. *Discov. Med.* **5**, 213–218.

Bernardello, M., Gora, R. J., Van Hage, P., Castro-Olvera, G., Gualda, E. J., Schaaf, M. J. M. and Loza-Alvarez, P. (2021). Analysis of intracellular protein dynamics in living zebrafish embryos using light-sheet fluorescence single-molecule microscopy. *Biomed. Opt. Express* **12**, 6205–6227.

Biteen, J. S., Thompson, M. A., Tselentis, N. K., Bowman, G. R., Shapiro, L. and Moerner, W. E. (2008). Super-resolution imaging in live Caulobacter crescentus cells using photoswitchable EYFP. *Nat. Methods* **5**, 947–949.

Brunsveld, L., Waldmann, H. and Huster, D. (2009). Membrane binding of lipidated Ras peptides and proteins — The structural point of view. *Biochim. Biophys. Acta BBA - Biomembr.* **1788**, 273–288.

Buning, R. and van Noort, J. (2010). Single-pair FRET experiments on nucleosome conformational dynamics. *Biochimie* **92**, 1729–1740.

Bryan, S. J., Burroughs, N. J., Shevela, D., Yu, J., Rupprecht, E., Liu, L.-N., Mastroianni, G., Xue, Q., Llorente-Garcia, I., Leake, M. C., et al. (2014). Localisation and interactions of the Vipp1 protein in cyanobacteria. *Mol. Microbiol.*

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263**, 802–805.

Canedo, A. and Rocha, T. L. (2021). Zebrafish (Danio rerio) using as model for genotoxicity and DNA repair assessments: Historical review, current status and trends. *Sci. Total Environ.* **762**, 144084.

Chen, C., Zong, S., Wang, Z., Lu, J., Zhu, D., Zhang, Y. and Cui, Y. (2016). Imaging and Intracellular Tracking of Cancer-Derived Exosomes Using Single-Molecule Localization-Based Super-Resolution Microscope. *ACS Appl. Mater. Interfaces* **8**, 25825–25833.

Detrich, H. W., III, H. W. D., Westerfield, M. and Zon, L. I. (2011). *The Zebrafish: Genetics, Genomics and Informatics*. Academic Press.

Donnert, G., Eggeling, C. and Hell, S. W. (2009). Triplet-relaxation microscopy with bunched pulsed excitation. *Photochem. Photobiol. Sci. Off. J. Eur. Photochem. Assoc. Eur. Soc. Photobiol.* **8**, 481–485.

Downward, J. (2003). Targeting RAS signaling pathways in cancer therapy. *Nat. Rev. Cancer* **3**, 11–22.

Filonov, G. S., Piatkevich, K. D., Ting, L.-M., Zhang, J., Kim, K. and Verkhusha, V. V. (2011). Bright and stable near-infrared fluorescent protein for in vivo imaging. *Nat. Biotechnol.* **29**, 757–761.

Fujiwara, T. K., Iwasawa, K., Kalay, Z., Tsunoyama, T. A., Watanabe, Y., Umemura, Y. M., Murakoshi, H., Suzuki, K. G. N., Nemoto, Y. L., Morone, N., et al. (2016). Confined diffusion of transmembrane proteins and lipids induced by the same actin meshwork lining the plasma membrane. *Mol. Biol. Cell* **27**, 1101–1119.

Funatsu, T., Harada, Y., Tokunaga, M., Saito, K. and Yanagida, T. (1995). Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution. *Nature* **374**, 555–559.

Garcia, G. R., Noyes, P. D. and Tanguay, R. L. (2016). Advancements in zebrafish applications for 21st century toxicology. *Pharmacol. Ther.* **161**, 11–21.

Gebhardt, J. C. M., Suter, D. M., Roy, R., Zhao, Z. W., Chapman, A. R., Basu, S., Maniatis, T. and Xie, X. S. (2013). Single-molecule imaging of transcription factor binding to DNA in live mammalian cells. *Nat. Methods* **10**, 421–426.

Giannone, G., Hosy, E., Levet, F., Constals, A., Schulze, K., Sobolevsky, A. I., Rosconi, M. P., Gouaux, E., Tampé, R., Choquet, D., et al. (2010). Dynamic superresolution imaging of endogenous proteins on living cells at ultra-high density. *Biophys. J.* **99**, 1303–1310.

Goodsell, D. S. and Olson, A. J. (2000). Structural symmetry and protein function. *Annu. Rev. Biophys. Biomol. Struct.* **29**, 105–153.

Gore, A. V., Pillay, L. M., Galanternik, M. V. and Weinstein, B. M. (2018). The zebrafish: A fintastic model for hematopoietic development and disease. *WIREs Dev. Biol.* **7**, e312.

Groeneweg, F. L., Royen, M. E. van, Fenz, S., Keizer, V. I. P., Geverts, B., Prins, J., Kloet, E. R. de, Houtsmuller, A. B., Schmidt, T. S. and Schaaf, M. J. M. (2014). Quantitation of Glucocorticoid Receptor DNA-Binding Dynamics by Single-Molecule Microscopy and FRAP. *PLOS ONE* **9**, e90532.

Ha, T., Ting, A. Y., Liang, J., Caldwell, W. B., Deniz, A. A., Chemla, D. S., Schultz, P. G. and Weiss, S. (1999). Single-molecule fluorescence spectroscopy of enzyme conformational dynamics and cleavage mechanism. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 893–898.

Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., van Eeden, F. J., Jiang, Y. J., Heisenberg, C. P., et al. (1996). The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development* **123**, 1–36.

Holm, T., Klein, T., Löschberger, A., Klamp, T., Wiebusch, G., van de Linde, S. and Sauer, M. (2014). A Blueprint for Cost-Efficient Localization Microscopy. *ChemPhysChem* **15**, 651–654.

Kadmiel, M. and Cidlowski, J. A. (2013). Glucocorticoid receptor signaling in health and disease. *Trends Pharmacol. Sci.* **34**, 518–530.

Keizer, V. I. P., Coppola, S., Houtsmuller, A. B., Geverts, B., van Royen, M. E., Schmidt, T. and Schaaf, M. J. M. (2019). Repetitive switching between DNA-binding modes enables target finding by the glucocorticoid receptor. *J. Cell Sci.* **132**, jcs217455.

Kim, S. H. (2018). TIRF-Based Single-Molecule Detection of the RecA Presynaptic Filament Dynamics. *Methods Enzymol.* **600**, 233–253.

Koopmans, W. J. A., Brehm, A., Logie, C., Schmidt, T. and van Noort, J. (2007). Single-pair FRET microscopy reveals mononucleosome dynamics. *J. Fluoresc.* **17**, 785–795.

König, I., Zarrine-Afsar, A., Aznauryan, M., Soranno, A., Wunderlich, B., Dingfelder, F., Stüber, J. C., Plückthun, A., Nettels, D. and Schuler, B. (2015). Single-molecule spectroscopy of protein conformational dynamics in live eukaryotic cells. *Nat. Methods* **12**, 773–779.

Kuhn, J. R. and Pollard, T. D. (2007). Single molecule kinetic analysis of actin filament capping. Polyphosphoinositides do not dissociate capping proteins. *J. Biol. Chem.* **282**, 28014–28024.

Kusumi, A., Fujiwara, T. K., Chadda, R., Xie, M., Tsunoyama, T. A., Kalay, Z., Kasai, R. S. and Suzuki, K. G. N. (2012). Dynamic Organizing Principles of the Plasma Membrane that Regulate Signal Transduction: Commemorating the Fortieth Anniversary of Singer and Nicolson's Fluid-Mosaic Model. *Annu. Rev. Cell Dev. Biol.* **28**, 215–250.

Kuzmenko, A., Tankov, S., English, B. P., Tarassov, I., Tenson, T., Kamenski, P., Elf, J. and Hauryliuk, V. (2011). Single molecule tracking fluorescence microscopy in mitochondria reveals highly dynamic but confined movement of Tom40. *Sci. Rep.* **1**, 195.

Kwakwa, K., Savell, A., Davies, T., Munro, I., Parrinello, S., Purbhoo, M. A., Dunsby, C., Neil, M. A. A. and French, P. M. W. (2016). easySTORM: a robust, lower-cost approach to localisation and TIRF microscopy. *J. Biophotonics* **9**, 948–957

Leake, M. C. (2013). The physics of life: one molecule at a time. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **368**, 20120248.

Lelek, M., Gyparaki, M. T., Beliu, G., Schueder, F., Griffié, J., Manley, S., Jungmann, R., Sauer, M., Lakadamyali, M. and Zimmer, C. (2021). Single-molecule localization microscopy. *Nat. Rev. Methods Primer* **1**, 1–27.

Lenn, T. and Leake, M. C. (2012). Experimental approaches for addressing fundamental biological questions in living, functioning cells with single molecule precision. *Open Biol.* **2**, 120090.

Lenn, T. and Leake, M. C. (2016). Single-molecule studies of the dynamics and interactions of bacterial OXPHOS complexes. *Biochim. Biophys. Acta* **1857**, 224–231.

Lieschke, G. J. and Currie, P. D. (2007). Animal models of human disease: zebrafish swim into view. *Nat. Rev. Genet.* **8**, 353–367.

Lippincott-Schwartz, J. and Patterson, G. H. (2003). Development and use of fluorescent protein markers in living cells. *Science* **300**, 87–91.

Liu, B., Zhang, T.-N., Knight, J. K. and Goodwin, J. E. (2019). The Glucocorticoid Receptor in Cardiovascular Health and Disease. *Cells* **8**, E1227.

Lommerse, P. H. M., Blab, G. A., Cognet, L., Harms, G. S., Snaar-Jagalska, B. E., Spaink, H. P. and Schmidt, T. (2004). Single-Molecule Imaging of the H-Ras Membrane-Anchor Reveals Domains in the Cytoplasmic Leaflet of the Cell Membrane. *Biophys. J.* **86**, 609–616.

Los, G. V., Encell, L. P., McDougall, M. G., Hartzell, D. D., Karassina, N., Zimprich, C., Wood, M. G., Learish, R., Ohana, R. F., Urh, M., et al. (2008). HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem. Biol.* **3**, 373–382.

Malumbres, M. and Barbacid, M. (2003). RAS oncogenes: the first 30 years. *Nat. Rev. Cancer* **3**, 459–465.

Marianayagam, N. J., Sunde, M. and Matthews, J. M. (2004). The power of two: protein dimerization in biology. *Trends Biochem. Sci.* **29**, 618–625.

Mashanov, G. I., Tacon, D., Knight, A. E., Peckham, M. and Molloy, J. E. (2003). Visualizing single molecules inside living cells using total internal reflection fluorescence microscopy. *Methods San Diego Calif* **29**, 142–152.

Metskas, L. A. and Rhoades, E. (2020). Single-Molecule FRET of Intrinsically Disordered Proteins. *Annu. Rev. Phys. Chem.* **71**, 391–414.

Miller, H., Zhou, Z., Shepherd, J., Wollman, A. J. M. and Leake, M. C. (2018). Single-molecule techniques in biophysics: a review of the progress in methods and applications. *Rep. Prog. Phys. Phys. Soc. G. B.* **81**, 024601.

Minsky, M. (1988). Memoir on inventing the confocal scanning microscope. *Scanning* **10**, 128–138.

Miyawaki, A., Shcherbakova, D. M. and Verkhusha, V. V. (2012). Red fluorescent proteins: chromophore formation and cellular applications. *Curr. Opin. Struct. Biol.* **22**, 679–688.

Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G. and Cormier, M. J. (1992). Primary structure of the Aequorea victoria green-fluorescent protein. *Gene* **111**, 229–233.

Reisser, M., Palmer, A., Popp, A. P., Jahn, C., Weidinger, G. and Gebhardt, J. C. M. (2018). Single-molecule imaging correlates decreasing nuclear volume with increasing TF-chromatin associations during zebrafish development. *Nat. Commun.* **9**, 5218.

Reyes-Lamothe, R., Sherratt, D. J. and Leake, M. C. (2010). Stoichiometry and architecture of active DNA replication machinery in Escherichia coli. *Science* **328**, 498–501.

Ritter, J. G., Veith, R., Veenendaal, A., Siebrasse, J. P. and Kubitscheck, U. (2010). Light sheet microscopy for single molecule tracking in living tissue. *PloS One* **5**, e11639.

Ross, J. L. and Dixit, R. (2010). Multiple color single molecule TIRF imaging and tracking of MAPs and motors. *Methods Cell Biol.* **95**, 521–542.

Rubin, H. (1990). The significance of biological heterogeneity. *Cancer Metastasis Rev.* **9**, 1–20.

Samatar, A. A. and Poulikakos, P. I. (2014). Targeting RAS-ERK signaling in cancer: promises and challenges. *Nat. Rev. Drug Discov.* **13**, 928–942.

Schaaf, M. J. M., Champagne, D., van Laanen, I. H. C., van Wijk, D. C. W. A., Meijer, A. H., Meijer, O. C., Spaink, H. P. and Richardson, M. K. (2008). Discovery of a functional glucocorticoid receptor beta-isoform in zebrafish. *Endocrinology* **149**, 1591–1599.

Schaaf, M. J. M., Koopmans, W. J. A., Meckel, T., van Noort, J., Snaar-Jagalska, B. E., Schmidt, T. S. and Spaink, H. P. (2009). Single-Molecule Microscopy Reveals Membrane Microdomain Organization of Cells in a Living Vertebrate. *Biophys. J.* **97**, 1206–1214.

Schwille, P., Haupts, U., Maiti, S. and Webb, W. W. (1999). Molecular dynamics in living cells observed by fluorescence correlation spectroscopy with one- and two-photon excitation. *Biophys. J.* **77**, 2251–2265.

Semrau, S. and Schmidt, T. (2007). Particle Image Correlation Spectroscopy (PICS): Retrieving Nanometer-Scale Correlations from High-Density Single-Molecule Position Data. *Biophys. J.* **92**, 613–621.

Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N. G., Palmer, A. E. and Tsien, R. Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nat. Biotechnol.* **22**, 1567–1572.

Shashkova, S. and Leake, M. C. (2017). Single-molecule fluorescence microscopy review: shedding new light on old problems. *Biosci. Rep.* **37**, BSR20170031.

Shashkova, S., Wollman, A. J. M., Leake, M. C. and Hohmann, S. (2017). The yeast Mig1 transcriptional repressor is dephosphorylated by glucose-dependent and -independent mechanisms. *FEMS Microbiol. Lett.* **364**,

Sheppard, C. J. R. (2017). Resolution and super-resolution. *Microsc. Res. Tech.* **80**, 590–598.

Shimomura, O., Johnson, F. H. and Saiga, Y. (1962). Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. *J. Cell. Comp. Physiol.* **59**, 223–239.

Soeller, C. and Cannell, M. B. (1999). Two-photon microscopy: imaging in scattering samples and three-dimensionally resolved flash photolysis. *Microsc. Res. Tech.* **47**, 182–195.

Sowa, Y., Rowe, A. D., Leake, M. C., Yakushi, T., Homma, M., Ishijima, A. and Berry, R. M. (2005). Direct observation of steps in rotation of the bacterial flagellar motor. *Nature* **437**, 916–919.

Stagge, F., Mitronova, G. Y., Belov, V. N., Wurm, C. A. and Jakobs, S. (2013). SNAP-, CLIP- and Halo-tag labelling of budding yeast cells. *PloS One* **8**, e78745.

Stoppin-Mellet, V., Bagdadi, N., Saoudi, Y. and Arnal, I. (2020). Studying Tau-Microtubule Interaction Using Single-Molecule TIRF Microscopy. *Methods Mol. Biol. Clifton NJ* **2101**, 77–91.

Stosiek, C., Garaschuk, O., Holthoff, K. and Konnerth, A. (2003). In vivo two-photon calcium imaging of neuronal networks. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 7319–7324.

Thompson, R. E., Larson, D. R. and Webb, W. W. (2002). Precise nanometer localization analysis for individual fluorescent probes. *Biophys. J.* **82**, 2775–2783.

Tinevez, J.-Y., Perry, N., Schindelin, J., Hoopes, G. M., Reynolds, G. D., Laplantine, E., Bednarek, S. Y., Shorte, S. L. and Eliceiri, K. W. (2017). TrackMate: An open and extensible platform for single-particle tracking. *Methods San Diego Calif* **115**, 80–90.

Tokunaga, M., Imamoto, N. and Sakata-Sogawa, K. (2008). Highly inclined thin illumination enables clear single-molecule imaging in cells. *Nat. Methods* **5**, 159–161.

Umemura, Y. M., Vrljic, M., Nishimura, S. Y., Fujiwara, T. K., Suzuki, K. G. N. and Kusumi, A. (2008). Both MHC class II and its GPI-anchored form undergo hop diffusion as observed by single-molecule tracking. *Biophys. J.* **95**, 435–450.

Uno, S.-N., Kamiya, M., Yoshihara, T., Sugawara, K., Okabe, K., Tarhan, M. C., Fujita, H., Funatsu, T., Okada, Y., Tobita, S., et al. (2014). A spontaneously blinking fluorophore based on intramolecular spirocyclization for live-cell super-resolution imaging. *Nat. Chem.* **6**, 681–689.

Willumsen, B. M., Christensen, A., Hubbert, N. L., Papageorge, A. G. and Lowy, D. R. (1984). The p21 ras C-terminus is required for transformation and membrane association. *Nature* **310**, 583–586.

Wollman, A. J. M. and Leake, M. C. (2016). Single-Molecule Narrow-Field Microscopy of Protein-DNA Binding Dynamics in Glucose Signal Transduction of Live Yeast Cells. *Methods Mol. Biol. Clifton NJ* **1431**, 5–15.

Yokota, H. (2020). Fluorescence microscopy for visualizing single-molecule protein dynamics. *Biochim. Biophys. Acta Gen. Subj.* **1864**, 129362.

Zacharias, D. A., Violin, J. D., Newton, A. C. and Tsien, R. Y. (2002). Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science* **296**, 913–916.

Zimmer, M. (2009). GFP: from jellyfish to the Nobel prize and beyond. *Chem. Soc. Rev.* **38**, 2823–2832.