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Shedding Light on Endocytosis With Optimized Super-Resolution Microscopy

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English summary

In this thesis we present the optimization of two aspects that affect the outcome of super-resolution microscopy images and the application of this optimized technique on the study of the fundamental cell process of endocytosis.

Super-resolution microscopy is one of the latest additions to a large list of microscopy techniques developed through the years, since the Dutch draper and scientist Antoni van Leeuwenhoek, and the English scientist Robert Hooke, built and used microscopes for the first time in the 17th century. Since then, and until the concepts of super-resolution were published and applied, the resolution limit of optical microscopy was ~200 nm, due to the diffraction barrier. In an optical microscope, a point source, such as a small emitting fluorophore in fluorescence based microscopy, appears as a disc of at least 200 nm of diameter, called Airy disk. When two point sources are closer together than this distance, their Airy disks merge and they cannot be seen as separate entities. In a biological sample, the many copies of the same protein labeled with a fluorescent dye also look like an Airy disk, making the distinction between single molecules impossible.

In the last few years, a number of approaches were developed to overcome this challenge, and they are grouped together as super-resolution microscopy. In this thesis, we focus on the further development and use of one of these approaches, called stochastic single molecule localization microscopy (SMLM). The base of stochastic SMLM is separating fluorescing molecules in time since they cannot be separated in space. For this, stochastic SMLM techniques exploit a chemical property of dyes that causes them to turn on and off under certain conditions. The on and off turning of molecules in time is colloquially called blinking, and it occurs stochastically. When only one fluorophore is on, the center of the Airy disk represents the original position of the molecule. Therefore, every time a fluorophore is on, its center is calculated to determine this original position. This process is repeated until all the centers of all the blinks/fluorophores have been calculated. In the end, an image is created by depicting all the calculated centers in a plane.

Here, we present the optimization of two aspects of stochastic SMLM in Chapters 2 and 3. First, we focused on improving the outcome of images obtained with fluorophores with suboptimal blinking. Blinking of fluorophores is the basis of stochastic SMLM and it determines how accurate they can be localized. However, every fluorophore blinks with different characteristics. In

some cases, suboptimal blinking of fluorophores that are on during long times gives rise to a type of background that is structured, because it comes from a structure in the sample that is labeled. Background of any kind can hamper the precision with which the centers of blinks are localized and can lead to images with artifacts. We show that structured background can be estimated and corrected with the application of a temporal median filter. This simple trick removes structured background, allowing the precise localization of molecules and generation of high-resolution artifact-free images.

We then focused on sample preparation for stochastic SMLM. The stochastic SMLM technique used in this thesis, GSDIM or dSTORM, is typically used on fixed samples, due to the long image acquisition times and the type of fluorophores used. Sample fixation procedures have been optimized and are well known to users of other microscopy techniques, but are only now beginning to be particularly evaluated for SMLM. Taking as a model actin and its binding proteins, we show the importance of specific fixation optimization for super-resolution microscopy. From electron microscopy experts, we know that the best fixative for ultra structural actin studies is glutaraldehyde. However, glutaraldehyde can induce loss and/or conformational changes in proteins, hampering the binding of antibodies used to label them for multicolor studies. We show that using paraformaldehyde, another type of aldehyde fixative, under proper conditions of temperature, pH and fixation times, the fine structure of actin is still maintained. Moreover, we found no detrimental effect of paraformaldehyde on any of the actin binding proteins tested.
Because of the infancy of super-resolution microscopy, the preparation of every new sample should be carefully optimized. For SMLM studies of actin and its binding partners, we show that paraformaldehyde fixation, and not glutaraldehyde fixation yields high quality artifact-free images.

The final goal of optimizing super-resolution microscopy is its application for the study of fundamental processes in cell biology. In Chapters 4 and 5, we use our optimized super-resolution microscopy to study endocytosis.

Endocytosis is a process through which cells internalize parts of their membrane, including proteins, many times by forming vesicles. There are many types of endocytosis. In clathrin mediated endocytosis, the internalized vesicles are coated by a protein named clathrin. In chapter 4, we present the results of the study of different clathrin coated structures, clathrin coated pits and flat clathrin plaques, that co-exist in cells. Using optimized super-resolution microscopy, we show that flat clathrin plaques are controlled by N-WASP and the Arp2/3 complex, actin polymerization activators.

Moreover, we found that flat clathrin plaques are platforms from where clathrin vesicles can be formed. Interfering with the dynamic actin polymerization

process via knock down of N-WASP or the Arp 2/3 complex, interfered with the dynamics of these vesicle platforms, and obstructed the internalization of two important membrane receptors: the Lysophosphatidic acid receptor 1 (LPAR1) and the epidermal growth factor receptor (EGFR). Interestingly, although endocytosis of both receptors was delayed, only the signaling pattern of LPAR1, and not that of EGFR, was affected, indicating that there is some level of specialization on plaque mediated endocytosis and signaling of particular receptors. More needs to be done to fully understand the role of actin controlled flat clathrin plaques in these important processes.

Internalization of receptors occurs after they are activated by their cognate ligands. Internalized receptors can no longer be activated on the membrane, which contributes in part to the cell´s signaling control. Therefore, inducing the internalization of overexpressed or mutated membrane receptors is an interesting therapeutic approach for cancer. A receptor that is commonly overexpressed or mutated in cancer is EGFR.

In Chapter 5, we show that endocytosis of EGFR in cells that overexpress it can be induced by Perifosine, a synthetic lipid. This internalization occurs in an unconventional manner, since EGFR does not get activated or ubiquitinated, steps necessary for its endocytosis. Moreover, Perifosine´s induced EGFR endocytosis prevents its activation by its cognate ligand, EGF. Interestingly, Perifosine does not induce EGFR endocytosis when the receptor is not overexpressed. In order to elucidate the mechanism of action of Perifosine and its specificity, we studied the organization of EGFR in the plasma membrane of these cells with super-resolution microscopy. We found that in cells that overexpress it, EGFR is oligomerized, while in cells without overexpression, EGFR is randomly distributed on the membrane. Although more studies need to be done, our preliminary results indicate that the specificity of Perifosine might be due to the contrasting distribution of EGFR on cells that express it at different levels.