Shedding light on endocytosis with optimized super-resolution microscopy

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Addendum
References

37 Huang, F. & Sorkin, A. Growth factor receptor binding protein 2-mediated recruitment of the RING domain of Cbl to the epidermal growth factor receptor is essential and sufficient to support receptor endocytosis. Molecular biology of the cell 16, 1268-1281, doi:10.1091/mboe.04-09-0832 (2005).


106 Wolter, S. et al. rapidSTORM: accurate, fast open-source software for localization


Addendum


177 Isogai, T., van der Kammen, R. & Innocenti, M. SMIFH2 has effects on Formins and p53 that perturb the cell cytoskeleton. Scientific reports 5, 9802, doi:10.1038/srep09802 (2015).


212 Clayton, A. H., Tavarnesi, M. L. & Johns, T. G. Unligated epidermal growth factor receptor forms higher order oligomers within microclusters on A431 cells that are sensitive to tyrosine kinase inhibitor binding. Biochemistry 46, 4589-4597, doi:10.1021/bi700002b (2007).


231 Wombacher, R. et al. Live-cell super-resolution imaging with trimethoprim conjugates.


Shedding light on endocytosis with optimized super-resolution microscopy

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.
Een nieuw licht op endocytose door middel van geoptimaliseerde super-resolutie microscopie.

Nederlandse samenvatting

In dit proefschrift beschrijven we de optimalisatie van twee aspecten van super-resolutie microscopie en het gebruik van deze geoptimaliseerde techniek in het bestuderen van het fundamentele cellulaire proces endocytose.

Super-resolutie microscopie is één van de jongste toevoegingen aan een lange lijst door de jaren heen ontwikkelde microscopie-technieken. Deze lijst begint in de 17e eeuw, toen de Nederlandse lakenhandelaar en wetenschapper Antoni van Leeuwenhoek en de Engelse wetenschapper Robert Hooke microscopen begonnen te bouwen en te gebruiken. Vanaf die begintijd, totdat de concepten van super-resolutie voor het eerst werden gepubliceerd, was de limiet van de haalbare resolutie van optische microscopie ~200 nm. Deze limiet komt door de diffractie barrière. In een optische microscoop wordt een puntbron, zoals bijvoorbeeld een enkel oplichtend fluorofoor in fluorescentiemicroscopie, afgebeeld als een schijf met een diameter van tenminste 200 nm, de zogeheten Airy schijf. Het centrum hiervan benadert de positie van de puntbron. Als twee puntbronnen echter dichter bij elkaar staan dan 200nm dan versmelten hun Airy schijven, met als gevolg dat ze niet afzonderlijk kunnen worden gedetecteerd. In een biologisch preparaat zijn de fluorescent gelabelde exemplaren van eenzelfde eiwit zo dicht bij elkaar dat ze overlappende Airy schijven vormen. Dit heeft als gevolg dat afzonderlijke moleculen niet kunnen worden onderscheiden.

In de laatste paar jaar zijn verschillende manieren ontwikkeld om deze uitdaging te overwinnen, allen onder dezelfde noemer: super-resolutie microscopie. In dit proefschrift focussen we op het verder ontwikkelen en gebruiken van één van deze benaderingen, namelijk stochastische single molecule localization microscopy (SMLM). Stochastische SMLM is gebaseerd op het scheiden van de fluorescente signalen in tijd, omdat dit op plaats niet mogelijk is. Om dit te bereiken benut SMLM een chemische eigenschap van specifieke kleurstoffen (dyes) die ervoor zorgt dat ze in bepaalde condities op een stochastische manier aan- en uitschakelen, in de volksmond ook wel blinking genoemd. Van elke fluorofoor die ‘aan’ is wordt het centrum van de Airy schijf berekend. Dit proces wordt herhaald totdat alle centra van alle blinks/fluoroforen bepaald zijn. Uiteindelijk wordt met al deze posities een super-resolutie beeld geconstrueerd.

In de hoofdstukken 2 en 3 presenteren we de optimalisatie van twee aspecten van stochastische SMLM. In de eerste plaats hebben we gefocust op het verbeteren van super-resolutie beelden die gemaakt zijn met fluoroforen die suboptimaal blinken. Het blinken van fluoroforen staat aan de basis van de stochastische SMLM techniek en bepaalt hoe nauwkeurig ze gelocaliseerd kunnen worden. Elke andere fluoroooor blinkt weer met verschillende karakteristiek. In sommige gevallen resulteert het blinken van fluoroforen die lang ‘aan’ staan tot een achtergrondsinaal. Omdat dit signaal niet uit het preparaat komt maar door het fluoroooor geproduceerd wordt,maakt dat niet willekeurig is, maar gestructureerd. Elk achtergrondsinaal vermindert de nauwkeurigheid van de localisatie van het echte signaal en kan leiden tot super-resolutiebeelden met artfacten. We laten zien dat gestructureerde achtergrond kan worden beoordeeld en gecorrigeerd door middel van...
een temporal median filter. Deze eenvoudige truc verwijdert gestructureerde achtergrond, waardoor precieze lokalisatie van moleculen mogelijk is en hiermee dus het genereren van een hoge-resolutie artefact-vrije beelden.

Daarna hebben we gefocust op sample preparatie voor stochastische SMLM. De stochastische SMLM techniek die gebruikt is in dit proefschrift, GSDIM of dSTORM, wordt vanwege de lange acquisitietijden en het type gebruikte fluoroforen meestal toegepast op gefixeerde preparaten. Algemeen bekende fixatieprocedures zijn geoptimaliseerd voor andere microscopietechnieken, maar worden pas recentelijk geëvalueerd voor SMLM. Door actine en actine-bindende eiwitten als model te nemen tonen we het belang aan van optimalisatie van de fixatie voor super-resolutie microscopie. Van elektronenmicroscopie-experts weten we dat het beste fixeermiddel voor ultrastructuurstudies glutaraldehyde is. Echter, glutaraldehyde kan conformationele veranderingen in eiwitten veroorzaken die de binding van antilichamen belemmeren. Terwijl deze antilichamen zijn nodig om de eiwitten te labelen met eerder genoemde dyes. We demonstreerden dat bij fixatie met paraformaldehyde, een ander fixatief, onder de juiste condities (temperatuur, pH en fixatietijd) de fijnstructuur van actine behouden blijft. Bovendien vonden we geen nadelig effect van paraformaldehyde op elk van de geteste actine-bindende eiwitten. Super-resolutietechnieken staan nog in de kinderschoenen, en daarom dient de preparatie van elk nieuw sample zorgvuldig te worden geoptimaliseerd. Voor SMLM studies aan actine en bindingspartners laten we zien dat paraformaldehyde-, en niet glutaraldehydefixatie, resulteert in hoge kwaliteit artefact-vrije afbeeldingen.

Het ultieme doel van het optimaliseren van super-resolutie microscopie is het toepassen ervan bij het bestuderen van fundamentele processen in de celbiologie. In Hoofdstukken 4 en 5 gebruikten we onze verbeterde super-resolutietechnieken voor onderzoek naar endocytose.

Endocytose is een proces waarbij cellen een gedeelte van hun membraan samen met eiwitten internaliseren, veelal door het vormen van vesicles. Er bestaan vele soorten endocytose. Bij één daarvan is het eiwit clathrin een belangrijke schakel, de geinternaliseerde vesicles zijn dan bekleed met clathrin, ook clathrin-coated genoemd. In Hoofdstuk 4 presenteren we de resultaten van een studie naar verschillende clathrin-coated structuren, clathrin-coated pits en platte clathrin plagues, die naast elkaar bestaan in de cel. Met behulp van geoptimaliseerde super-resolutie microscopie laten we zien dat platte clathrin plagues gereguleerd worden door N-WASP en het Arp2/3 complex, beide activators van actin polymerisatie. Daarnaast hebben we gevonden dat clathrin plagues platformen zijn van waaruit clathrin vesicles kunnen worden gevormd. Het beïnvloeden van de dynamische actine polymerisatie via het uitschakelen van N-WASP of het Arp2/3 complex leidde tot veranderingen in de dynamica van deze platformen. Daarnaast blokkeerde het de internalisatie van twee belangrijke membraanreceptoren: de lysofosfatidinezuur-receptor 1 (LPAR1) en de epidermale groeifactor-receptor (EGFR). Hoewel endocytose van beide receptoren werd vertraagd, veranderde opmerkelijk genoeg alleen het signalingspatroon van LPAR1, en niet dat van EGFR. Dit resultaat impliceert een zekere specifichtheid in plaque-gemedieerde endocytose en signaaltransductie van bepaalde receptoren. Verder onderzoek zal moeten uitwijzen wat precies de rol is van de door actine gereguleerde platte clathrin plagues bij deze belangrijke processen.

Internalisatie van receptoren vindt plaats nadat ze geactiveerd zijn door hun verwante
liganten. Reeds geïnternaliseerde receptoren kunnen niet meer op het membraan worden geactiveerd, wat deels bijdraagt aan de regulatie van celsignalering. Het induceren van internalisatie door middel van overexpressie van mutante membraanreceptoren is daarom een interessante therapeutische benadering voor kanker. Een receptor die vaak tot overexpressie komt of gemuteerd is bij kanker is EGFR.

In Hoofdstuk 5 laten we zien dat endocytose van EGFR, in cellen waarin deze tot overexpressie komt, veroorzaakt kan worden door perifosine, een synthetisch lipide. Deze internalisatie geschiedt op een onconventionele manier, omdat EGFR niet wordt geactiveerd of geubiquitinileerd; iets wat normaliter voor endocytose benodige stappen zijn. Bovendien verhindert perifosine-ge induceerde endocytose activering door zijn verwante ligant, EGF. Wanneer de receptor niet tot overexpressie wordt gebracht leidt dit interessant genoeg niet tot internalisatie van EGFR. Om het werkingsmechanisme en de specificiteit van perifosine op te helderen hebben we de organisatie van EGFR in het plasmamembraan van deze cellen onderzocht met super-resolutie microscopie. We hebben gevonden dat in cellen met overexpressie EGFR oligomeren vormt, terwijl in cellen zonder overexpressie EGFR willekeurig is verdeeld over het membraan. Hoewel er meer onderzoek zal moeten worden gedaan, impliceer onze eerste resultaten dat de specificiteit van perifosine wellicht wordt veroorzaakt door de uiteenlopende verdeling van EGFR op het membraan van cellen met verschillende expressieniveaus.
Curriculum Vitae

Daniela Monica Leyton Puig was born on September 22, 1979 in Cochabamba, Bolivia. She obtained her high school diploma from Saint Andrew’s School in La Paz, Bolivia, after which she enrolled in the Computer Science Bachelor program at the “San Pablo” Catholic University in La Paz, Bolivia. In 2002 she moved to Madrid, Spain where she obtained a Bachelor in Biology and Masters’ Degree in Biomedicine at the Complutense University. She continued her education and obtained a second Masters’ degree in Molecular Biotechnology at the University of Barcelona in Barcelona, Spain. In Barcelona, she worked as a researcher for the Spanish Council for Scientific Research (CSIC) in the Institute of Molecular Biology of Barcelona (IBMB) under the supervision of dr. Isabel Uson, where she worked in the crystallization and functional characterization of AtzR, a bacterial DNA binding protein. During this period she collaborated with the group of dr. Fernando Govantes in the Department of Molecular Biology and Biochemical Engineering at the University Pablo de Olavide, in Sevilla, Spain, where she worked for a short period. In December 2011, she began her PhD training in the Biophysics group of prof. dr. Kees Jalink at the Netherlands Cancer Institute (NKI) in Amsterdam. Her project was part the STW nanoscopy consortium funded by the STW technology foundation, which included several universities and companies committed to further develop super-resolution microscopy. The results of her contribution to the development of the technique and its application in cell biology research are described in this thesis.
Publication list

**Leyton-Puig D**, Isogai T*, van den Broek B, Klarenbeek J, Janssen H, Jalink K, Innocenti M.
* These authors contributed equally
Flat clathrin lattices are dynamic actin-controlled hubs for clathrin-mediated endocytosis and signalling of specific receptors
Manuscript submitted

GDE3 suppresses urokinase receptor activity through GPI-anchor cleavage
Manuscript submitted

Glycerophosphodiesterase GDE2 Promotes Neuroblastoma Differentiation through Glypican Release and is a Marker of Clinical Outcome.

**Leyton-Puig D**, Kedziora KM, Isogai T, van den Broek B, Jalink K, Innocenti M.
PFA fixation enables artifact-free super-resolution imaging of the actin cytoskeleton and associated proteins.

Rapid Remodeling of Invadosomes by Gi-coupled Receptors: Dissecting the role of Rho GTPases.

Isogai T, van der Kammen R, **Leyton-Puig D**, Kedziora KM, Jalink K, Innocenti M.
Initiation of lamellipodia and ruffles involves cooperation between mDia1 and the Arp2/3 complex.

Argenzio E, Margadant C, **Leyton-Puig D**, Janssen H, Jalink K, Sonnenberg A, Moolenaar WH.
CLIC4 regulates cell adhesion and β1 integrin trafficking.

Hoogendoorn E*, Crosby KC*, **Leyton-Puig D***, Breedijk RM, Jalink K, Gadella TW, Postma M.
* These authors contributed equally
The fidelity of stochastic single-molecule super-resolution reconstructions critically depends upon robust background estimation.

Nieuwenhuizen RP, Lidke KA, Bates M, **Leyton-Puig D**, Grünwald D, Stallinga S, Rieger B.
Measuring image resolution in optical nanoscopy.
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