Unraveling Intermediate Filaments
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Pijnlijk beworteld in een bloedige grond,
groei ik met takken gemaakt van dromen.
CHAPTER 6

General Discussion
The Nobel Prize 2014 in chemistry was awarded to Betzig, Hell and Moerner for the development of Super Resolution (SR) microscopy: ingenious technology to surpass the diffraction limit of light. During last years, SR has found its way not only into the nomination list of scientific awards and prizes, but also to the microscopy facilities around the world as a standard technique for biology research. Single Molecule Localization microscopy (SMLM) is one of the SR techniques and has already been used to address several questions in the biomedical field [154, 155] [156] [157] [158] [159] [160]. However, the quality of the images produced with this technique can still be improved. In this regard, two considerable refinements have been covered in this thesis. A newly developed buffer system and a new imaging chamber now allow multi-color imaging with an extended field of view in 2D and 3D. Furthermore, we have developed new approaches to quantify co-localization and co-alignment in super resolved images. These developments have been applied in several studies, including those on intermediate filaments (IF) in human cells. With these new technological improvements of SMLM we have discovered that hemidesmosomes are formed around non-looping single keratin filaments in human keratinocytes.

A major focus of this thesis is the optimization of the buffer composition. Image quality relies on blinking behavior of the dye, which can be dramatically affected by buffer composition and oxygen level in the environment. Oxygen plays dual roles in On/Off blinking [85, 86]. On one hand, it produces radical derivatives (for example singlet oxygen) upon laser illumination which may lead to irreversible bleaching. On the other hand, oxygen is a triplet state quencher and therefore reduces triplet-state-induced bleaching [161]. In conclusion, the oxygen level in the buffer should be kept under strict controls. This task proved to be challenging because the often-used enzymatic systems are unstable and their by-products can be detrimental to the preparation [83, 87]. The complexity of this issue is best illustrated in multicolor imaging, where the imaging buffer should be tuned for each individual dye type and therefore, a compromise has to be set.

In this thesis, we have presented a new buffer system: OxEA (primarily composed of Oxyrase with a tailored PH). OxEA enhances blinking of the most-common dyes in localization based microscopy, such as Alexa 647, Alexa 532 and Alexa 488, all at the same time. Cyanine-dyes blink optimally in the absolute absence of oxygen, while Rhodamine-dyes perform poorly without oxygen. As a weak oxygen scavenger, OxEA reduces the oxygen concentration (not complete depletion) and creates an ideal oxygen balance for both Cyanine-dyes and Rhodamine-dyes (Chapter 2). Additionally, OxEA doesn’t acidify the environment, is stable for a longer period of time and is suitable for tissue SMLM imaging.

For dyes that perform the best in the absence of oxygen (mainly Cyanine-based dyes), we have designed a new method to block oxygen influx entirely. The Oxygen Tight Chamber (OTC) is an imaging chamber completely impermeable to oxygen. Remarkably, it can retain low oxygen levels up to several months after sealing. In an oxygen-free environment, the
photo bleaching rate decreases. Consequently, higher numbers of photons can be registered and localization precision is raised considerably (Chapter 2). With such a reduced bleaching rate, a larger field of view is attainable in 2D (image stitching) and 3D (extended depth of view).

Another issue that limits SMLM and has a negative influence on image quality is mechanical drift of the sample in the lateral and axial directions. New stages have been constructed [123] and several post processing techniques have been designed to mitigate the mechanical drift [162, 163]. However, the imaging chamber itself has never been identified as a major source of drift. We have shown that the curing procedure of glass-to-plastic bonding can be a source for drift in glass bottom dishes, since the unmatched materials (glass and plastic in this case) remain under stress. OTC is the first dedicated chamber for SR imaging that can reduce the drift by using an alternative glass bonding procedure. In 2016, WillCo Wells Company commercially launched our developed chamber (OTC) to the market (WillCo-dish NANO, glass bottom, GWSB-3512N).

Another aspect that needs careful attention is interpreting of super resolved images in a quantitative manner. Image analysis routines that have been developed for conventional optical microscopy do not apply for SMLM results due to fundamental differences between conventional and SMLM images. Where conventional optical microscopy images consist of pixels, indicating the local intensity of light from fluorescent molecules, SMLM images are reconstructions of discrete coordinates and therefore appear dotty. This dottiness though is an inherent characteristic of SR images hinders their interpretation and complicates colocalization analysis in dual color imaging.

In conventional dual color imaging, each pixel has two intensities, one for each label. In Manders/Pearson methods, the overlap between labels can be numerically expressed by a coefficient, based on pixel intensities. In SMLM, however, pixel intensities have been scaled by the number of corresponding and above-threshold localizations in each pixel. These localizations, representing the positions of the molecules, essentially never overlap, regardless of whether they locate close to each other or not. Therefore, conventional methods for colocalization measurement cannot be applied directly and should be adapted first to the SMLM technique.

Colocalization between two protein species in SR images has been studied carefully by several research groups around the world. The results can be classified into two different approaches [164] [165]:

1. Coordinate based analysis: a distance-dependent cross-correlation approach to determine whether two sets of localizations are in close proximity [92] [93] [94].
2. Pixel based analysis: to discover the level of colocalization in recognized shape [166] [155].
The coordinate based analysis is one of the most employed methods to analyze colocalization in SR microscopy and is available in open source post processing packages [109]. This approach, however, is not always effective and is inherently prone to artifacts. For example, coordinate based analysis on a small area of a SR image may result in a false negative signal, whereas sampling of a larger area in the same image can show a perfect colocalization [167]. To avoid such artifacts, we adopted a simple alternative approach to quantitate colocalization in this thesis. To investigate the nearness of two different labels, we have developed a pixel-based analysis method called Proximity Mapping (PM) that visualizes the proximity of the labels across the image (Chapter 3).

Measuring colocalization in SR imaging can be extremely challenging especially studying specific structures. In this respect, not only the local proximity of the molecules, but also the relative positions within a structural context should be carefully considered. To answer questions like “are strings of red molecule located in the neighborhood of green-molecule strings?” or “are these strings of red molecules parallel to strings of green molecules?” we devised an alternative method, named co-orientation analysis. Co-orientation considers the alignment of the structure as an extra information dimension. Computed in sub-regions of a single cell, co-orientation can be plotted as a function of (1) distance and (2) alignment angle between localizations and filaments (Chapter 5).

Colocalization analysis is not the only requirement that needs to be met in a SR post processing routine. Optical distortions, such as chromatic aberration, need to be compensated and SR images can be highly improved by robust background subtraction [110]. To accelerate post processing of SR images, all the routinely applied packages should be synchronized. A user-friendly interface, as a universal post processing unit, may enhance efficiency in image refinement.

Considerable efforts have been made to improve SMLM images during the last years. In theory, the obtainable resolution (localization precision) in super resolved images is a few nanometers. Nevertheless, the real resolution has been practically limited by the size of primary/secondary antibodies. Normal antibodies are massive in size (~150 kDa [168]), and add significant uncertainty to the position of the epitope (molecule). During the last years, several studies have been conducted to overcome this limitation. A few representative examples are nano-bodies [169], cameld antibodies [168, 170], Fab fragments [171], tetracysteine labels [172] and Click chemistry [173] that all aim to minimize the size of the labels. However, these replacements are either very expensive (not affordable for daily use in cell biology) or their quality is lower than traditional antibodies (for example, they have lower affinity for the target protein). Designing smaller and cheaper antibodies with higher affinity would revolutionize the SMLM microscopy.

Due to recent optimizations of the preparation and improved analyzing methods, multi-color imaging has become eminently practical and the produced images can be interpreted
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We have applied these new techniques and methods to study the interaction between IFs and other cellular components. In this respect, Hemidesmosome (HD, a complex adhesion-structure in human skin), and keratin IF (a major IF sub-type with an ability to connect to HD), both come into focus in this thesis.

Keratin IFs have an average diameter of only 10 to 15 nm and thus cannot be studied in depth by conventional confocal laser scanning microscopy. SR 3D imaging of keratin in human keratinocytes confirms that the keratin organization is significantly different at the cell periphery compared to perinuclear regions. Thick keratin filament bundles extend throughout the cytoplasm and create an elaborate network of fine filaments at the cell periphery (Chapter 4).

We have shown that keratin intermediate filaments may end at the cell periphery, where they often have a striped pearl string-like appearance. The keratin pearls have a predominant size of 60 - 80 nm and are less obvious in the perinuclear region of the cell. Super resolved images show that in areas where keratin assemble or disassemble, such as in the lamella of migrating cells, keratin pearls show wider spacing with gaps of about 60 -100 nm between pearls. It is currently unclear what caused pearl-string appearance and what is the reason that pearl spacing appears to be wider in the lamella. We have only carried out a preliminary analysis of this new observation. Pearl string filaments can be observed with different antibodies, in different cell types, and with different IF proteins. Whether this appearance reflects epitope masking by accessory proteins, or un-condensed filaments, or perhaps both, remains to be determined in future research.

Keratin branching have also been reported in this thesis. Our analysis revealed that branching does not occur predominantly at a fixed angle, like in Arp2/3-mediated actin branching. Initially we speculated that specific accessory proteins may be involved in mediating branching. However, this hypothesis was not further addressed in detail. Interestingly, during preparation of the manuscript for chapter 4, Nafeey et all presented Electron Microscopy data showing branching of keratin in pressure frozen keratinocytes. These authors also observed branching in vitro in highly purified preparations of keratin 8/18 filaments expressed in Escherichia coli, raising the interesting possibility that branching is an inherent capacity of IF. Whether in cells branching involves accessory proteins awaits further studies.

Our data show that at the periphery of keratinocytes, where HDs are formed, keratin filaments are decorated with plectin and β4 integrin. We have shown that plectin and β4 integrin localize mainly alongside the keratin filaments rather than directly under them as was hypothesized in text books. Interestingly, BP180 and BP230 are also associated with keratin filaments in the cell periphery, albeit they have a different characteristic distribution, in which several BP180 molecules surround tight clusters of BP230 molecules. The association of the α6β4/plectin patches with BP180/BP230 and our analysis by PM suggests that β4
integrin and BP180 are located in close proximity in mature HDs. The latter finding is in line with previous studies by biochemical assays; though it has never been observed directly at such high resolution. Due to our improvements in tissue staining and by OxEA buffer, HD organization has been verified in human skin sections by SR microscopy (Chapter 3).

In this work, we have also studied several other proteins that are essential for HD formation, and presented their interaction in vivid details. It is important to remember that these details were observed in fixed cells. Although single time-point observations in dead cells gives a lot of information, it would be revealing to observe the dynamic processes at super resolved level in living cells. To do this, one can apply other SR microscopy methods that support live cell imaging, such as SIM or live-cell PALM.

In the scope of this thesis, we did not study so-called Focal Contact (FC), the other adhesion structure in human keratinocytes. It is undisputable that both FCs and HDs share some proteins to regulate keratinocytes motility [174]. Indeed, both of them appear involved in skin wound healing [175]. Nonetheless, whether these two functionally-distinct complexes interact directly or by competing the same proteins remains debated [175]. To start, CD151 (a tetraspanin family member) can be a major player in the communication between FC and HD ultrastructural adhesion complexes. Monitoring CD151 with super resolved accuracy may provide valuable evidences of its role as a functional linker [48, 174]. Our preliminary unpublished data show close spatial proximity between CD151 and other proteins involved in FCs (such as α3) and HDs (such as β4 integrin). Further studies are needed to unravel the role of IFs in the interaction between these macromolecular assemblies.

Another question is “what the role of tetraspanin web in the formation of HDs is in general?” SR is the right tool to derive new insights to this question. Additionally, other tetraspanin family members (CD9 and CD81) can be studied to understand the interaction between tetraspanin network and HDs proteins in meticulous details.

The next topic covered in this thesis is the orientational alignment between vimentin IF and MT. The interaction between MT and vimentin and its regulation has been received a close attention in early and recent studies. Though it has been discovered that several proteins are involved in the connection between IFs and microtubules, [22, 176] its significance is still elusive. Our preliminary observation by SR has presented a clear spatial proximity between vimentin and MT at the level of single filaments, as well as bundles. Applying co-orientation measurement on several cell lines, we have quantitatively shown that interaction between vimentin and MT in nano scale is not a generic property of those filaments in all cells, but it crucially depends on the cell type. Moreover, we have observed that the co-orientation of MT and vimentin is predominant in the cell periphery and not in the perinuclear region of the cells (Chapter 5).

In biology, co-orientation is a satisfactory solution to measure the proximity between two single filaments at a nano-scale, and two cytoskeleton meshworks at a larger scale (Chapter
5). MT and vimentin interact in both scales, is shown by biochemical assays and proven by SR microscopy. Biological manipulation of the link between microtubules and vimentin, and applying high resolution microscopy afterwards can disclose extra information about this co-operation.

SMLM has improved dramatically since its invention and a large part of that has taken place in the last few years. In this context I can only present a small sample of the rapid developments that are published regularly. New software routines were developed for complex analysis and quantification of SR images [94] [177]. Three dimensional SMLM imaging has become a matter of routine to address biological questions [178]. Cameras have become fast and reliable, and the acquisition time was reduced dramatically [179]. These improvements have been used to study a variety of cellular structures, and the results have on several occasions necessitated updates in our biological models and understanding [180] [181]. The discoveries made possible by SMLM techniques have been well received and now also increasingly become cited by main-stream follow-up biological papers [182]. Finally, live cell imaging has become in reach, thanks to the new fast cameras, to improved fluorescent proteins that blink well, and to new small labels that are cell-permeant. With these developments SMLM can become a standard tool in the cell biology lab, and perhaps also for disease pathology [181] SMLM offers endless possibilities for scientists, as the future of microscopy has become entangled with nano-imaging.

Concluding remarks
During this research, we have identified several critical limiting factors in localization based super resolution microscopy across the imaging spectrum: from preparation and imaging condition to post processing, quantification and visualization. We have found practical solutions and one of them even has been introduced to the microscopy market (WillCo-dish NANO, glass bottom, GWSB-3512N). Next, we have applied these improved aspects to challenging questions in biology. The results in this thesis provide novel insights into the intermediate filaments structure and their essential role in organizing adhesion structures such as hemidesmosomes. We could successfully update the textbook-model for the spatial organization of hemidesmosome components thanks to detailed SR images. This study also has contributed to the understanding of interactions between intermediate filaments and other cytoskeletal components such as microtubules.

The last word
Massive technical improvements in the field of nanoscopy have made it possible to study healthy and diseased cells in exceptional detail. I sincerely hope that SR microscopy can contribute in our battle against cancer.