Rennen en gaan,
met beide handen krachtig slaan,
in de maanlichte hemel, tegen de maanronde bal,
met veel vreugde van die vurige aanval.
CHAPTER 1

General Introduction
1. PREFACE

The human body has similarities to an advanced and organized community of people, where each individual should dutifully contribute to the same ultimate goal: a healthy society. However, a few individuals may on purpose disrupt the structure of society, leading to chaos, rebellion and war. In medical science, one form of rebellion of cells in the body is named “cancer”.

While a lot is known about what transforms a healthy cell to a cancer cell, much more is still to be learned. A key role is reserved for genes and their transcription, but it is broadly agreed that different cellular structures may also contribute to the transformation and disease progress. Furthermore, these structures may reflect the diseased state of the transformed cells. For example, certain subgroups of intermediate filaments (IF; see further) that are involved in cell adhesion and migration can be expressed differently in cancer cells.

In the malfunctioning cell, some of these IFs may become over-expressed [1] and others may be completely absent, depending on the cancer type and host organ [2]. There are also IFs that show an altered organization in cancer cells. Such IF expression even is used as a diagnostic biomarker for cancer diagnosis [3, 4]. Clearly it is essential to understand the structure and organization of IFs, as well as the way they interact with cell adhesion molecules and other cellular components. However, much of these structures are far too small to be resolved by light microscopy. To achieve detailed knowledge on the IF cytoskeleton and its interactions, we have employed super resolution (SR) microscopy, specifically Ground State Depletion followed by Individual Molecular return (GSDIM) microscopy [5] [6] [7].

GSDIM presents a giant leap forward in resolution and yet, like other new methods, it needs further improvements. In this thesis, we introduce several improvements, including a new imaging buffer for GSDIM to obtain two- and three color images, improvements aimed at increasing preparation longevity, which is useful for “image stitching” in 2D and 3D, improvements in the mechanical stability of the preparation and improvements in the quantitative analysis of the images. Thanks to these technical refinements, GSDIM has become a more reliable instrument for advanced cell biology and we were able to use it successfully to investigate intermediate filaments.

2. BIOLOGY

a) Intermediate filaments, a brief overview

There are three classes of cytoskeletal filaments actin based Micro Filaments (MFs), tubulin-based Microtubules (MTs) and Intermediate Filaments. Among these classes, IF are certainly the most diverse. Unlike MTs (diameter 25 nm) and MFs (diameter 7 nm), the average
diameter of IFs varies between 10 to 12 nm, to frame the concept of “intermediate”. IFs play major roles in cell: from bearing tension and maintaining cell shape and rigidity, to anchoring cells to the extracellular matrix via integrins [8]. Furthermore, a large number of associated proteins bind to IFs and therefore, IFs provide attachment sites for those other protein assemblies.

IFs are classified into six subgroups based on amino acid sequences [9] (Table 1). Type I and Type II IFs are acidic and basic (or neutral) keratins, respectively, and are produced by different types of epithelial cells. Type III IFs, are expressed in different cell types: vimentin can be found in fibroblasts and white blood cells and desmin in smooth muscle cells. Two other specific types (glial fibrillary acidic protein and peripherin) are only expressed in glial cells and neurons of the peripheral nervous system. Type IV IFs are neurofilament proteins which exist in three different types: light, medium and heavy (NF-L, NF-M, NF-H) variants. They are particularly abundant in axons of neurons. The last member of this group is α-internexin, which is an essential protein in neuron development. Type V, so called nuclear lamins, rather than being a part of the cytoskeleton, merely exist in nuclear envelopes and are the underlying structure of nuclear membranes. Nestin belongs to the Type VI group of IFs and it is the only one located in the central nervous system (Table 1) [10].

**Table 1. Intermediate filament proteins**

<table>
<thead>
<tr>
<th>Type</th>
<th>Protein sub type</th>
<th>Size (kDa)</th>
<th>Expressed in</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Acidic keratin, neutral or basic keratin</td>
<td>40-60</td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>II</td>
<td>Neutral or basic keratins</td>
<td>50-70</td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>III</td>
<td>Vimentin, desmin, glial fibrillary acidic protein and peripherin</td>
<td>51-54</td>
<td>Fibroblast, White Blood cells, Muscle cells, Glial cells and Peripheral neurons</td>
</tr>
<tr>
<td>IV</td>
<td>Neurofilament proteins (NF-L, NF-M, NF-H), α-internexin</td>
<td>66-200</td>
<td>Neurons</td>
</tr>
<tr>
<td>V</td>
<td>Nuclear lamins</td>
<td>60-75</td>
<td>All cell types</td>
</tr>
<tr>
<td>VI</td>
<td>Nestin</td>
<td>200</td>
<td>Stem cells in nervous system</td>
</tr>
</tbody>
</table>

**b) IF, chemical and molecular structure**

Although there is a large diversity among IF proteins, they share a similar assembly plan [11]. A polypeptide α helical domain forms the central rod region. N-terminal and C-terminal domains, present at opposite sides of the α helical domain, serve as ‘head’ and ‘tail’ respectively. Two proteins form a coiled-coil dimer with a length of 45 nm, which can be considered the basic building block for IF assembly [12]. In the next step, the dimers assemble into tetramers and align in an anti-parallel fashion to make filaments. This way, they grow along the filament axis in a non-polarized manner [12]. The properties of the coil structure on one hand and the amino acids in the tail domain on the other hand, determine
the basic dissimilarities among different IF proteins and specify the unique characteristics of IFs in structure and function [11] (Fig. 1). The structure of intermediate filaments (especially keratin) has been studied and presented in chapter four of this thesis.

![Fig. 1. Assembly plan of filamentous intermediate filaments.](image)

Each dimer consists of two polypeptides (B) that wrap around each other with their rod domain (orange). Dimers associate in an antiparallel mode (C), while the N-terminus of two dimers locate on the opposite sites of the resulting tetramer, and the same statement is valid for their C-terminus. Tetramers assemble end to end and grow laterally (D) to make proto intermediate filaments. Eight of a proto filaments anneal around each other and form one single filament (E).

Among all these IFs, three were studied in more detail in this thesis: vimentin, keratin and lamins.

a) Vimentin

Vimentin, a type III intermediate filament, is highly expressed in mesenchymal cells and is a marker of epithelial to mesenchymal transition [13]. In general, vimentin is responsible for maintaining cell integrity. Furthermore, it is associated with cell migration and wound healing in cultured cells [14]. Vimentin knockout mice appear normal; however, they are defective in wound healing. The embryonic fibroblasts of these mice don’t migrate, unless vimentin is reintroduced; in that case the motility of these cells can be completely rescued [15]. Recently, researchers have shown a significant association between vimentin expression and cell polarity as well as motility [16]. Interestingly, vimentin is newly
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distributed and overexpressed in various epithelial-derived cancers such as prostate cancer, gastrointestinal tumors, CNS tumors, breast cancer, malignant melanoma and lung cancer [2]. Furthermore there is lines of evidence support the involvement of vimentin in tumor growth rate and tumor genetic events via various signaling pathways. Therefore vimentin could be a potential target in cancer therapy [17].

There is strong evidence of association between vimentin and other cellular components such as MF [15, 18], myosin [19] and mitochondria [20]. Importantly, MTs (the cellular highways for vesicle transportation) interact with vimentin [21-23]. Though this interaction has been observed by conventional light microscopy and Electron Microscopy (EM), its significance is yet elusive. This interaction will be studied in more details in chapter 2 of this thesis.

b) Keratin

Keratin provides mechanical resilience to the cell [24]. In addition, the keratin network reorganizes quickly in response to various stimuli. For example, it has been shown that keratin carries out a protective function under induced cytoskeletal changes and mechanical stress. Recently, roles in other basic cellular activities, such as growth, proliferation, organelle transport [25] have also been attributed to Keratin.

Keratin plays an indirect role is in cell adhesion and migration. It has been shown that keratin represses cell migration in keratinocytes by mediating hemidesmosome (HD) integrity [26]. HDs are nano-structures which firmly adhere keratinocytes in the epidermis to the basement membrane and dermis in skin. Dermis and epidermis are the outer and inner layers of human skin, respectively and they are separated by the basement membrane which consists of extracellular matrix protein. Thus HDs support resilience against mechanical forces by adhering skin layers. Therefore, HDs play a critical role in skin integrity [8, 27].

It has been hypothesized that keratin binds to plectin (a member of the plakin family of cytoskeletal linker proteins) via its C-terminus domain [28-31]. Since plectin is the cytolinker between \( \alpha_6\beta_4 \) integrin and keratin IF, any change of the keratin network may affect HD structure [32, 33]. To elaborate on the link between Keratin and cell motility, it is essential to understand the structure of HD in fine detail.

In EM images, HDs look like dense plaques at which Keratin filaments are anchored. When explored by biochemistry, two types of HDs are distinguished based on their protein constituents: (1) type II HDs, the most abundant type, mainly exists in stratified and pseudostratified tissue and contains the integrin \( \alpha_6\beta_4 \) and plectin. (2) Type I HDs are present in the simple epithelial cells such as those lining the intestine [34], and additionally accommodate proteins known as BP antigens: BP180 and BP230 [28, 29, 35].

Integrin \( \alpha_6\beta_4 \) and plectin are the core of each HD, in both type I and II. In mice, targeted disruption of the genes for these proteins causes neonatal trauma-induced blistering [36-
In humans, mutations in the genes for α6, β4 or plectin lead to a skin-blistering disorder referred to as Epidermolysis bullosa [39-41]. This clearly indicates that α6β4 and plectin are two main building blocks for HD creation, and their loss causes dramatic effect on the structure.

The direct interaction between α6β4 and plectin occurs through the cytoplasmic domain of β4 and the N-terminal region of plectin [42-44]. A primary β4 binding site is located within the actin-binding domain (ABD) of plectin. When the β4 subunit is bound to plectin, interaction with F-actin is prevented [32, 43]. This indicates that HDs are indirectly involved in actin dynamics and consequently in cell motility.

Three extra components exist in type I HDs, compared to Type II HDs:

1. Bullous Pemphigoid antigen BP180 (collagen type XVII or BPAG2): this is a type II transmembrane collagen that has affinity for collagen IV and laminin-332.
2. Bullous Pemphigoid antigen BP230 (BPAG1): this protein is encoded by an epithelial splice variant of the dystonin gene, another member of the plakin family. It acts primarily as a linker between BP180 and IFs [45-47].
3. The tetraspanin CD151: a cell surface protein with four transmembrane domains. It associates with α6β4 and regulates the spatial organization of HDs [48].

Both BP180 and BP230 can interact with integrin β4 subunits and are therefore critical players in the formation of HDs in cultured keratinocytes. In both humans and mice, relatively mild blistering disorders can result from mutations in the genes BP180, BP230 and CD151 [8, 49-53].

A model for the organization of the HD components has been deduced from biochemical assays with keratinocytes. These cells are derived from EB patients and from in vitro protein-protein interaction data [44]. The model suggests a three-layered structure, in which the three transmembrane proteins are connected to the IF system via two plakin members. However, it is not known how integrin α6β4 and BP180 are spatially organized relative to one another and to the plakin members. Neither is it clear how the keratin-IFs are anchored to newly formed HDs, and whether they are directly involved in the organization of the hemidesmosomal structure. These questions have been addressed in chapter 3 of this thesis.

c) Nuclear Lamins

Nuclear lamins are Type V intermediate filaments. These small fibrils make a mesh of filaments between the inner nuclear membrane and peripheral heterochromatin, the so called lamina [54]. Typically, two lamine sub-types are in the lamina meshwork of mammalian cells: (1) A-type lamins (lamin A and lamin C) and (2) B-type lamins (lamin B1 and Lamin B2).
[55, 56]. Lamin A and lamin B1 interact with each other mono-typically and hetero-typically in lamina meshwork, as was shown by Fluorescence Resonance Energy Transfer (FRET) [57]. Recently, it has become apparent that, though interactive and present in the same structure, A-type and B-type lamins form separate filamentous networks in the lamina [58, 59]. This finding has been recently verified by SR microscopy, showing that lamin A, C, B1 and B2 are organized in distinct fibrillar meshworks in mouse embryo fibroblasts [60].

As a framework for nuclear envelop structure, the lamina is involved in the regulation of complex nuclear activities such as DNA replication, DNA repair, transcription and chromatin reorganization [61, 62]. Cell differentiation is another process in which a subgroup of lamins has recently been implicated [63]. There are numerous studies linking lamins to diseases and aging. In 1999, a group of researchers proved that Autosomal Dominant Emery-Dreifuss Muscular Dystrophy (AD-EDMD) is a result of a mutation in the gene that encodes lamin A and Lamin C.s [64]. In less than two decades, hundreds of similar mutations have been identified as involved in causing several human diseases [65], including certain cancers.

It has been shown that lamin expression levels are altered in many tumor cells [66]. This change is so dramatic that it can be used to diagnose the level of tumors progression. In addition, there is a clear link between the morphology of the nuclear structure (and the lamina as a part of it) and cancer [66]. In spite of the corroborative evidence, lamins’ reorganization in cancer cells is not fully explored. Neither is clear how this reorganization influences the gene expression machinery and chromatin remodeling. DNA adenine methyltransferase identification (DamID) combined with microscopy techniques, can provide vivid details on chromatin structure and its binding sites to the lamina, so called Lamina Associated Domains (LADs) [67].

The DamID technique is based on the fact that adenine methylation does not occur endogenously in most eukaryote cells. However, such a methylation is widespread in bacterial genomes as a part of their restriction modification system [68]. In DamID, the *Escherichia coli* DNA adenine methyltransferase (Dam) is fused to the nuclear protein of interest, in this case lamin proteins. In the native binding sites of chromatin to the nuclear lamina, Dam adds one methyl group at position 6 of the adenine in GATC motif, and produces N6-methyladenine (m6A). Hence, close proximity of DNA and lamina generates a uniquely mark on the chromatin, the methylation tag. This tag is detectable using methylation sensitive restriction enzymes: DpnI and/or DpnII. These enzymes have been fused to GFP via their N-terminus for observation purposes. Under the epifluorescence microscope, LADs look like scattered bright regions in the lining of the nuclear envelope.

DamID techniques have been successfully employed to study lamina and chromatin interaction in several cases. [69, 70]. We studied the interaction between chromatin and DNA in fine details by SR microscopy. Some of the images from this work have been presented in the image smorgasbord in the Appendix.
3. TECHNIQUE

a) Diffraction limits the resolution in light microscopy.

The crucial role which light microscopy plays in broadening our knowledge of cells is apparent. Yet traditional light microscopy forms have limited resolving power due to diffraction of light. Light diffraction is a phenomenon that occurs when a parallel light beam passes an aperture. After passing the aperture the light beam deviates in many directions forming a typical diffraction pattern with a maximum in the centre and several concentric sub-maxima around the central maximum.

The dimension of the central maximum depends on the size of the aperture; the smaller the aperture, the larger the diameter of the central maximum. A microscope objective is an optical element with refractive and diffractive properties. The most important refractive property is that it directs a parallel light beam towards a single infinitely small focal point. But an objective is also an aperture and therefore diffraction of light causes the direction of light to change according to the laws of diffraction. This is the reason why the source of the light may be infinitely small while the size of the image of the point source is finite: it is “diffraction limited”. Thus, the resolution of the image of an object is limited by diffraction. The resolution of the image is directly related to the resolving power of the microscope as defined by Abbe’s Law:

\[ d = \frac{\lambda}{2n \sin \theta} \]

Where \( d \) is the smallest distance between two point sources of light that can be resolved by the optical system, \( \lambda \) is the wavelength of the light, \( n \) is the refractive index of the medium and \( \theta \) the opening angle of the objective which depends on the focal distance and the aperture of the objective. This equation clearly shows that the resolution is directly related to the dimension of the aperture of the objective. The term \( nsin\theta \) in the denominator, called Numerical Aperture (NA) is an important property of a microscope objective and defines the resolution of the microscope. Although Abbe’s Law describes a fundamental limitation in light microscopy, there are several new technologies that circumvent this law, collectively called Super Resolution Microscopy.

b) Bypassing the diffraction limit is feasible.

Super resolution is a widely used term to specify a group of microscopy techniques that circumvent the diffraction limit. Nowadays, many of these approaches are routinely used in biological studies and there are more approaches theoretically explained in the literature [71, 72]. Here, we discuss the most well-known techniques briefly:
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i. **Near Field methods:**
   - **Total Internal Reflection (TIRF):** In this technique the sample is not fully exposed to the excitation light, but only the part of the sample that is very near to the surface of the cover glass. This specific illumination is achieved by an optical effect called total internal reflection. Fluorophores in a very thin section (about 200nm) of the sample are excited and will emit light which is detected by a camera. TIRF is a super resolution technique in the axial direction only, and can be combined with other techniques which provide super resolution in the lateral direction [73].
   
   - **Near field Scanning Optical Microscopy (NSOM):** The simple trick of SNOM is to make the size of the excitation source and the size of the light detector smaller than the wavelength of light. In practice, a tiny detector (e.g., an optical fiber with nanometer-sized tip) is placed very close to the specimen surface of the cell and collects undiffracted light. By this approach, the resolution can be enhanced down to 2-5 nm [74] but the technique can only be applied for imaging of the surface of the cell.

ii. **Far Field methods:**
   - **4Pi microscopy:** This technique is based on the formation of diffraction patterns in the axial direction of the optical system. This is achieved by illumination of the sample with coherent light from two opposite directions by two opposing lenses. Due to the diffraction effects in the axial direction, this technique has a strongly improved axial resolution [5].
   
   - **I^5M microscopy:** I^5M is similar to the 4Pi technique; nevertheless the light source is an arc lamp instead of laser illumination [75, 76]. The excitation light in I^5M is passed through a polarizer before reaching the specimen. Placing two light sources on both sides produces an axial interference pattern and as a consequence sharpens the full-width, half max (FWHM) of the conventional Point Spread Function (PSF). Light collection, on the other hand, is through a system identical to 4Pi.
   
   - **Confocal microscopy and Re-scan Confocal Microscopy (RCM):** This technique is based on excitation in a single point by a focused laser beam that moves through the sample in a scanning manner. Fluorescence from the sample is detected after the emission light passes a pinhole. Although confocal microscopy is most often not regarded as a super-resolution technology, its lateral resolution surpasses the diffraction limit as defined by Abbe’s Law.
When this pinhole is small enough, lateral resolution is improved by a factor of $\sqrt{2}$. The drawback of a small detection pinhole is that the light efficiency of the confocal microscope is very low. This problem is solved in Re-scan Confocal Microscopy (RCM) where the emitted light is directed via an extra set of scanning mirrors onto a sensitive camera. By control of the scanning mirrors a $\sqrt{2}$ improvement of the lateral resolution can be achieved without reduction of the diameter of the pinhole. This means that RCM is a technique with improved lateral resolution and enhanced sensitivity [72].

- **Stimulated Emission Depletion (STED):** This method overcomes the diffraction barrier by confinement of the excitation volume in a fluorescent sample and therefore, narrowing the excitation PSF [5]. To achieve this, the light sources are defined as two synchronized pulsed laser beams. The first laser beam, the so-called excitation laser, illuminates the fluorophore on the sample in a scanning manner with a normal PSF. The second laser beam, called the depletion laser, has a wavelength matching the emission of the fluorophore and its focus spot has the shape of a doughnut. The light of the depletion laser drives the fluorophores into the ground state by stimulated emission and therefore depletes spontaneous emission. The fluorophores in the center of the illuminated region are excited by the excitation laser but are not illuminated by the depletion laser (intensity equals zero in the center of the doughnut). Therefore, spontaneous fluorescence emission can be detected only in the center of the depletion doughnut. In this way the effective size of the excitation PSF is strongly reduced. More recently, this trick has also (simultaneously) been applied in axial direction, delivering much improved resolution in 3D.

- **Structured Illumination Microscopy (SIM):** In SIM, the sample is illuminated with a pattern of fine stripes. This illumination pattern interferes with features in the object and results in generation of Moiré effect. By changing the illumination pattern in time (e.g. rotating or shifting) a series of images with modulated information can be registered. This information then can be decoded to produce the reconstructed SIM images. SIM improves the lateral and axial resolution up to two-fold [77, 78].

- **Photo Activated Localization Microscopy (PALM):** PALM uses genetic fusion constructs of fluorescent proteins that exist initially in a dark state and can be activated by light. In this technique, a wide-field low-power laser beam (often UV) sends a small subset of molecules to the On-state stochastically. A high
power laser beam is used to pinpoint the position of the On-state molecules precisely and also converts them to the Off-state again via photobleaching. The is repeated many cycles and the final super resolved image is the sum of multiple localizations. Fluorophore intermittency (blinking) is not essential in PALM, since the fluorescent proteins bleach after the first round of excitation. However, repeatable cycle between On- and Off-states may increase image quality [7].

- **Stochastic Optical Reconstruction Microscopy (STORM):** Similar to PALM in principle, STORM relies on chemical fluorescent dyes. A wide field illumination activates and bleaches photoswitchable organic fluorophores stochastically in repeated cycles, while dedicated powerful Software localizes the fluorophores by Gaussian fitting algorithms. In STORM, however, dye-pairs consisting of two spectrally different fluorophores are used to enable repeated cycling. The first photo-switchable member of this pair, so-called activator, facilitates activation of the second member, so-called reporter fluorophore. The reporter then cycles between On- and Off- states iteratively. A powerful Software locates reporter’s position with nanometer accuracy [79]. STORM can be carried out with Blue-Red and UV-Red dye pairs; though in all cases the readout is Red, which enables multi-color imaging without chromatic aberrations.

- **Ground state depletion followed by individual molecule return (GSDIM) / direct Stochastic Optical Reconstruction Microscopy (dSTORM):** This is the main technique which has been used in this thesis, it will be more extensively discussed in the coming paragraphs. PALM, STORM, dSTORM and GSDIM are the key members of the “Single Molecule Localization based Microscopy” (SMLM) technique. SMLM is extremely powerful, reaching localization precisions down to a few nm.

c) dSTORM/GSDIM: a closer look

GSDIM/dSTORM is a technique applicable for synthetic fluorophores; however, use of dye-pairs is not necessary. In dSTORM or GSDIM, reversible and controllable switching of the dyes happens directly as a consequence of laser illumination (Fig. 2). First of all, a high power laser beam excites almost all the fluorophores from the ground state to the first excited state at the same time. Then, excited molecules come back to the ground state via two different pathways: most of the excited molecules fall back via the fluorescent pathway and emit photons, so called molecules in On-state. This process is fast and takes place in a few nanoseconds. Some molecules, however, transit to a dark state or triplet state that
may last from milliseconds to many seconds. In this state dyes are in a temporary \textit{Off}-state but not bleached. Because the dark state is so long, eventually (several seconds after starting the experiment) almost all molecules will be in the dark state. The few remaining fluorophores in the \textit{On}-state are individually visible due to their bright fluorescence and they can be registered precisely with a sensitive Electron Multiplying Charge Coupled Devise (EM-CCD) or scientific Complementary Metal-Oxide Semiconductor (sCMOS) camera. These molecules are then converted into the dark state, while other molecules stochastically return to the \textit{On}-state again. The molecules that continuously transit between \textit{On/Off}-states appear to blink and are known as \textit{blinkers}. The precise position coordinates of \textit{On}-state molecules are calculated by Gaussian computer fitting. Iteration of this process allows numerous fluorophores to be localized and the final SR image can be reconstructed with registered coordinates of the localizations. Therefore, the resulted SR image is no longer intensity based, but a collection of coordinates registered with extreme precision [80, 81]. The localization precision is directly proportional to the square root of emitted photons and therefore, sufficient numbers of photons are needed for a high resolution image (Fig. 3).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fluorophore_blinking_diagram}
\caption{Energy/Jablonski diagram of fluorophore blinking. In the bright On-state, fluorophores can be excited (Ex) from the ground state (S\textsubscript{0}) to the excited singlet state (S\textsubscript{1}). From there, they may either relax to the ground state by emitting a quantum of light (hv) or alternatively, they may undergo intersystem crossing to the dark triplet state (T\textsubscript{1}). From the triplet state, molecules may return to the ground state or progress to a second, long-lived dark state (D), e.g. through redox reactions. Direct transfer from the excited singlet state to the dark state has also been reported [82] (dashed arrow). Molecules in the dark state may return to the ground state by inverse redox reactions, or alternatively, by exposure to near-UV radiation (back-pumping). S\textsubscript{0} and S\textsubscript{1} are called On-states, while T\textsubscript{1} and D are Off-states. With most molecules in the Off-state, it is possible to detect the few remaining bright fluorophores individually in the preparation. Dark states may last between milliseconds and minutes, whereas triplet states may last microseconds at ambient oxygen levels.}
\end{figure}
Blinking crucially depends on the buffer in which the dye is immersed. This buffer usually contains oxygen scavengers and aliphatic thiols in order to control the dark state. Improved blinking can also be induced by irradiating the preparation with weak UV light, so-called back-pumping.

As mentioned, GSDIM and dSTORM can reach localization precisions down to a few nm. Note, however, that the coordinates reflect the position of the fluorescent label. In case of labeling with a primary antibody + fluorescently labeled secondary antibody, the epitope may be up to 15 nm away from the label. This restricts the resolution and therefore much efforts are aimed at obtaining smaller, yet specific, labels.

One advantage of SMLM is that it can be carried out on relatively simple instruments. In essence, a mechanically very stable wide-field light microscope is used, equipped with strong lasers, a fast EMCCD or sCMOS camera, and fast acquisition Software. Often, SMLM is combined with TIRF illumination, which helps rejecting out-of-focus signal but has the disadvantage that only a thin layer close to the coverslip can be imaged.

Alternatively, 3D images may be acquired in either of two manners. First, SMLM inherently has Z-axial sectioning capabilities because *On-state* dyes that are > 1 micrometer above or below the focus position will appear blurry and dim. They are therefore rejected by the fitting algorithm. A course 3D image can therefore be assembled from several conventional SMLM images taken at different Z position. A second, more precise approach makes use of an astigmatic (cylindrical) lens which is placed in front of the camera. This cylinder lens distorts (enlarges) the images of the blinking molecules either in width or in height, depending on whether the molecule is above or below the exact focal plane. These distortions can be detected by fitting algorithms and they yield axial (Z-plane) information with about 50 nm precision.

On the other hand, SMLM has posed several challenges, both for experimentation and for data analysis, as will be detailed below.
Fig. 3. Image reconstruction in SMLM microscopy. (A) Fluorophores in the On-state are visible individually due to their bright fluorescence. One fluorophore in the On-state is magnified in the orange square. Though smaller than 10 nm, the photon distribution pattern of one single fluorophore has spread to an area of about 6 X 6 pixels (pixel size= 10nm) due to diffraction. The position of fluorophores in On-state can be registered precisely by Gaussian fitting algorithms (white crossed circles). (B) The final SR image is the result of thousands of frames, in which each frame contributes a collection of registered localization coordinates. An example SR image is built up from left to right in this image panel. Each panel contains data from 3000 extra frames compared to the one located to its left. Image becomes sharper and brighter from left to right. (C) A TIRF image (left) and corresponding SR image (right). The selected area surrounded with white boundaries is the same magnified area in (A) and (B).
d) Buffer composition: an important player in SMLM image quality

Blinking (switching of fluorophores between On/Off-states) is the essence of the SMLM technique. As mentioned above, the blinking properties of dyes can be heavily influenced by adding or removing reductants and oxidants to the imaging buffer/embedding system [83]. In other words, to obtain high quality super resolution images, the buffer plays a crucial role. However, conventional buffers suffer from several shortcomings. For example, oxygen scavengers like Glucose Oxidase are widely used in buffer compositions to prevent the dyes from bleaching [84]; nevertheless, they produce reactive oxygen species (ROS) as a byproduct. The dyes either terminally bleach or do not blink perfectly in the presence of reactive oxygen species [85, 86]. Additionally, most conventional buffers have a limited lifespan. As a classic example, the often-used Gloxy buffer [83, 87] stops functioning in less than one hour because it is inherently unstable. Finding the optimum buffer for one single dye is challenging enough, and it becomes even more complicated when other dyes are used in addition. In the case of Gloxy, it is a well-matched buffer for the Cyanine-based dyes (e.g., Cy5 and Alexa 647) [83]. However, Rhodamine-based dyes (e.g., Alexa 555 and Alexa 488) do not blink well in Gloxy. Conversely, in buffers that make Rhodamine-based dyes blink, Cyanine-based dye do not perform brilliant. Thus, it has been impossible to obtain excellent two- or three-color images. The shortcomings of existing buffers and our proposed solution will be discussed in detail in chapter 2.

e) Bleaching severely restricts SR imaging.

Fluorophores can reversibly switch between excited (On) and ground (Off) state for several times and emit photons. In SMLM, image quality also relies on the number of On/Off cycles (see STORM/GSDIM). It takes several minutes before enough blinking events can be collected for a high quality image. In more elaborate applications, such as image stitching and 3D imaging, much more time is needed, and the dyes may bleach before enough events have been detected.

Bleaching is a result of the reaction between fluorophores and molecular oxygen [88]. In this chemical reaction, therefore, oxygen levels are a crucial factor. It has been shown that the photobleaching rate becomes much lower by protecting dyes against the environmental molecular oxygen [89] and oxygen scavenging protocols have been described that are now in common use to address this oxygen issue [90, 91]. However, during the experiment there is continued oxygen influx that will eventually saturate the oxygen scavengers. This topic will be discussed in more detail in chapter 2.

f) Colocalization: an obsolete concept in SMLM

In conventional microscopy images, colocalization of two molecular species is often quantified using Pearson or Manders coefficients, i.e. by calculating the overlap of the
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intensity between two colors. In SR images, where individual molecules are displayed in principle as “dots” (coordinate pairs with in principle nearly infinite precision and an associated localization uncertainty of ~5-10 nm), exact overlapping of molecules is extremely rare. For example, molecules in a large molecular complex would be seen to colocalize with normal light microscopy but they could be more than 50 nm apart in the complex, leading to zero colocalization in a SR image. Thus, it is not possible to apply traditional colocalization methods to SR images. Several alternative methods have been proposed to replace colocalization in SR images [92-94]; yet, it is important to further develop dedicated strategies to quantify closeness for SR based microscopy, and we contributed several new methods as described in chapter 3 and chapter 5 in more detail.

4. THESES OUTLINE

IFs, intermediate size cytoplasmic /nuclear filaments, are involved in numerous activities in the cells: for example, sustaining mechanical stress, organizing cellular adhesive structures and gene transcription and regulation. Furthermore, there is a link between IFs and diseases, in particular certain types of cancer. In this thesis, the substructure of IFs as well as the way they cooperate with other cellular components comes into focus. To employ two-color SR microscopy, we first improved the sample preparation and buffer composition.

Chapter 2 describes a novel buffer, designed for the multi-color SMLM technique. We examined several buffering systems and have developed a new buffer that works equally well with Cyanine- and Rhodamine-based dyes. We call our new buffer OxEA. In this chapter, we show that OxEA offers several benefits, it:

- Depletes oxygen to the desired level,
- Has a longer usable life time,
- Does not acidify,
- Provides a single preparation that is compatible with multiple dyes, thus it is suitable for multi-color imaging.

In chapter 2, we also introduce and characterize new improved imaging chambers designed specifically for SR microscopy. These chambers address major challenges in SR, namely mechanical drift and oxygen influx. Working with an industrial partner, WillCo Wells (Amsterdam), we have produced and characterized very low-drift glass bottom culture dishes. In addition, we introduced a method to effectively prevent oxygen influx. With these improvements it becomes possible to carry out very long experiments and do image stitching (mapping the whole cell by combining several high-resolution sub-images) and extensive 3D imaging (with or without cylindrical lens) because it allows drift-free imaging for extended times. These improvements also enabled using the same preparation for up to 2 months.
depending on the antibodies. Using OxEA and the OTC, we have studied various aspects of the organization of keratin intermediate filaments and hemidesmosomes (chapter 3, 4 and 5).

In **Chapter 3** we show that:

- In the cell periphery, nascent HDs associate with individual keratin filaments, where β4 integrin is distributed along, and not under keratin filaments.
- The HD plaque protein plectin interacts simultaneously and asymmetrically with β4 integrin and keratin.
- BP180 and BP230 are characteristically arranged with BP180 molecules surrounding a central core of BP230 molecules.

In chapter 3, we also investigate the structure of HDs in skin sections. The SR images of tissue directly show that plectin occupies the position in between β4 integrin and the keratin IF system, as was expected from biochemical assays in vitro. In conclusion, chapter 3 highlights the power of SR imaging in elucidating biological structures and it shows the importance of keratin IF in orchestrating HDs in human skin.

The last part of chapter 3 focuses on a practical way to visualize molecular vicinity via pixel mapping of different color labels. This Proximity Map (PM) highlights the places that two labels are in close vicinity. We extensively tested different methods for creating such PM and we found out a fast and user friendly method to calculate the nearness of the pixels occupied by different colors. With PM we then demonstrated that the transmembrane proteins BP180 and β4 integrin are present in the same HDs.

In **Chapter 4** we present two new observations made by SR on intermediate filaments, along with a preliminary analysis. First we show that keratin filaments often display a distinct pearl string-like appearance with alternating bands of intense and weak staining. Interestingly, this phenotype is predominantly observed at the periphery of the cells and in motile lamella-like structures. Preliminary quantification indicates that pearls spacing is at about 60-80 nm, which is close to the length of Unit-Length Filaments (ULFs). We also found that keratin filaments display prominent branching. Branching has been reported for actin filaments, but at the onset of this study, no branching capacity had been proposed for any intermediate filament. Unlike actin branching, keratin branching appears to have little preference for specific angels. Keratin is also seen to form nano-loops, which demonstrates the high flexibility of the elementary keratin filaments.

Further studies of IFs and other filamentous systems, made possible by our technical improvements, reveal an intriguing alignment between vimentin IFs and MTs. With the superior resolution of SR imaging, vimentin can be seen to run parallel to (or sometimes wrap around) MTs. Although PM is a straightforward method to measure vicinity/colocalization of two proteins, it does not take into account structural information, such as the direction of filaments. To quantitatively describe the co-alignment of the two filamentous structures, in
Chapter 5 we collaborated with researchers from Delft University of Technology to devise a new image analysis method to analyze parallel filaments in SR images. In this chapter, we measure co-orientation between vimentin IF and MT in three assessed cell lines.

We worked on many more projects than cannot be described in detail in this thesis. Some of these projects were collaborations with other groups that sought to include SR imaging into their own work. In other cases, SR allowed us to observe intriguing new details in cells that we simply did not have time to elaborate on. Some of our detailed images are presented in the image smorgasbord, which is presented as an appendix at the end of the thesis. These images also attract attention to the pure beauty which is knitted with science and nature, at this nano-scale.