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Vernieuwingsimpuls
Innovational Research Incentives Scheme
Grant application form (full proposal) 2018   **Vici scheme**

Ultra-resolution with visible light   *Bernd Rieger*

Registration form (basic details)

1a. Details of applicant
-Title: Prof. Dr.
-First name: Bernd
-Initials: B.
-Surname: Rieger
-Address for correspondence:

         Faculty of Applied Sciences
         Delft University of Technology
         Lorentzweg 1, 2628 CJ Delft

-Preference for correspondence in English: no
-Telephone: +31(0)152788574
-Mobile phone: +31(0)614390021
-Email: b.rieger@tudelft.nl
-Website: http://homepage.tudelft.nl/z63s8/

1b. Title of research proposal
Ultra-resolution with visible light

1c. Scientific summary of research proposal

Optical nanoscopy is a revolutionary technique for studying biological structure and function at the nanometer scale using fluorescent labels. Localization microscopy offers a ~10x better resolution than conventional diffraction-limited microscopy, improving the resolution from ~250 to 30 nm. This improvement, however, is still a factor of 10 short from the molecular-scale ultra-resolution (~1 nm) that is needed to unravel the structure and composition of protein complexes with regular or impaired functionality. For instance, the 3D composition of the nuclear pore complex is still much debated despite its important function as gateway between cell nucleus and cytoplasm and its dysfunction being connected to several autoimmune diseases.

Fluorescence imaging at cryogenic temperatures enables high localization precision (sub-nm) as more photons can be acquired from the labels, but the actually achievable 3D resolution is still not in the desired 1-nm range. This is due to unavoidable incomplete fluorescent labelling and inherently poor axial resolution in microscopy. Currently cryo-electron microscopy does provide near-atomic resolution but cannot reveal biomolecular composition.

I propose to combine the development of new prior-knowledge-driven image reconstruction schemes and a new cryogenic fluorescence technique to enable 3D isotropic 1-nm resolution in localization of single biological molecules. To this end, I will (1) combine information from many chemically identical complexes into one single reconstruction in a manner that compensates for incomplete fluorescent labelling, and (2) realize an experimental setup and methodology for imaging at cryogenic temperatures with two aberration-corrected objective lenses on opposite sides of the sample. The latter will offer isotropic localization precision in the sub-nm range and improved axial resolution through interferometric detection. Importantly, my cryogenic fluorescence approach can be combined with cryo-electron microscopy in one workflow, allowing for the first time both structural characterisation and functional identification of interacting protein complexes at 1-nm resolution.

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1d. Keywords
3D ultra-resolution microscopy, structural biology, cryo fluorescence, multi-view geometry, image reconstruction

1e. Current institution of employment
Faculty of Applied Sciences
Delft University of Technology
Lorentzweg 1
2628 CJ Delft, The Netherlands

1f. Prospective host institution (If known)
As above

1g. NWO assessment committee (Choose <u>one</u>)

Applied and engineering sciences (AES)	**X**
Health Research and Development (ZonMW)	
Science domain (ENW)	
Social Sciences and Humanities (SSH)	
Cross-domain committee (DO)	

1h. Main field of research

Code	Main field of research
16.20.00	Software, algorithms, control systems
	Other fields of research
12.20.00	Nanophysics/technology
14.90.00	Technology, others
21.90.00	Life sciences, other

1i. Public summary of your research proposal
Zooming in on the building blocks of life, one at a time.
Prof.dr. Bernd Rieger (m), TUD – Imaging Physics
Microscopes can zoom deeper and deeper into biological matter. They can even discern single molecules using fluorescent labels. Researchers solve a persistent shortcoming of fluorescence microscopy and devise a new microscopy methodology to image molecular structures at the millionth of a mm scale, opening new ways to answer fundamental questions about the building blocks of life.

Inzoomen op de bouwstenen van het leven, één voor één.
Prof.dr. Bernd Rieger (m), TUD – Imaging Physics
Microscopen kunnen steeds dieper inzoomen op biologische materialen. Door middel van fluorescerende labels kunnen ze zelfs enkele moleculen onderscheiden. Onderzoekers lossen een hardnekkig tekortkoming van fluorescentiemicroscopie op en ontwikkelen een nieuwe microscopiemethode om moleculaire structuren van een miljoenste mm groot te bekijken, om uiteindelijk fundamentele vragen over de bouwstenen van het leven te kunnen beantwoorden.

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Research proposal

2a1. Overall aim and key objectives

Super-resolution microscopy has revolutionized nano-scale imaging by making it possible to image sub-cellular structures at ~30-nm length scales with visible light [Betzig2006, Rust2006, Hell2009, Huang2010, Klein2014]. The Nobel Prize in chemistry was awarded in 2014 to Hell, Betzig and Moerner for this innovation. A new leap in resolution of a factor of 10 would open up the exciting possibility of imaging *within* the macromolecular complexes that make up the machinery of life.

Single-molecule localization microscopy (SMLM) is the most widely applied type of optical super-resolution microscopy. Its final resolution is, however, limited by [Nieuwenhuizen2013] **(1)** incomplete fluorescent labelling, which is unavoidable in practice [Sauer2017, Wang2014]; **(2)** the limited number of photons that can be harvested from a single fluorescent molecule [Dempsey2011, Wang2014]; and **(3)** the poor resolving power along the microscope's optical axis that is inherently 3-5x worse than in the image plane [Rieger2012]. **The goal of my project is to overcome these three connected challenges and obtain isotropic 1-nm resolution in 3D.**

Together with my group I have made exciting progress in cryogenic fluorescent imaging and pioneered the fusion of information from multiple-image acquisition in the past years. Based in these results, I am convinced that *computational microscopy*, that is, the correct combination of microscopic imaging with dedicated computational methods can have a dramatic impact on the achievable image — its resolution and useful information content. The possibility to do structural biology with light instead of exclusively with complex electron microscopy is in itself already thrilling. Even more so is the combination of both techniques, offering the prospect of adding direct functional identification of protein complexes to the "grey" images of electron microscopy. In particular, I envision the ability of resolving the composition of the structure of certain nucleoporins (Nups) in the Nuclear Pore Complex (NPC), where 3D information on the nm-scale is required to map the densely packed proteins.

The project is divided into two work packages:

- **WP1: 3D data fusion.** We will develop ways to optimally combine information from many chemically identical complexes into one single reconstruction. Building on my very recent work in 2D for data fusion [Heydarin2018], this will compensate for incomplete fluorescent labelling. In addition, we will develop ways to include potential symmetry of complexes and automatic multi-class identification to accommodate *a-priori* unknown biological heterogeneity.
- **WP2: 4pi cryo.** We will build a 4pi cryogenic super-resolution fluorescence microscope. We will realize localization-based ultra-resolution using 4pi detection for isotropic localization precision and polarization control to induce sparsity in combination with polarized STED. We will add aberration control optics into both emission arms of the microscope in order to obtain axial and in-plane resolution down to an unprecedented 1 nm, i.e. achieve true ultra-resolution.

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In the following sections, I will sketch the context, requirements and benefits of super-resolution microscopy at 1-nm resolution in 3D, followed by the key objectives that I plan to achieve. Subsequently, I will outline my research strategy that is based on advances in algorithms, my expertise in optical imaging, and strong collaborations with experimental partners.
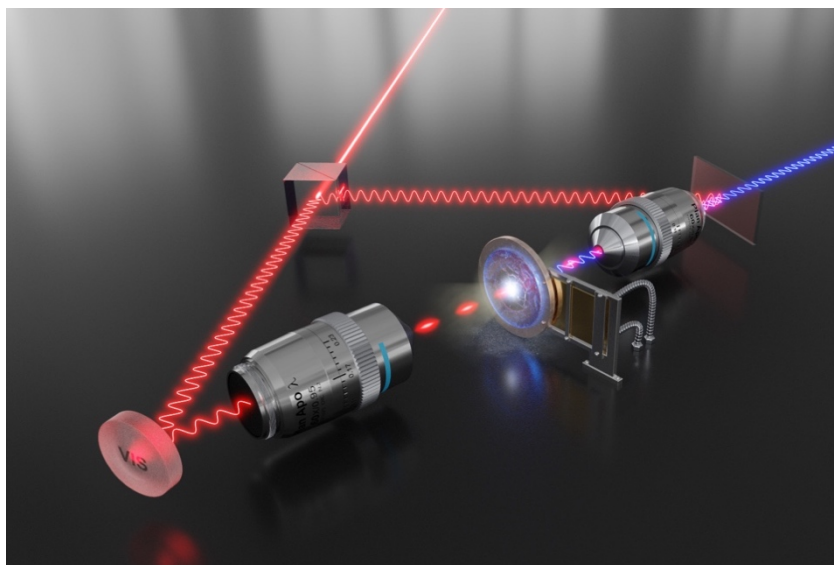
Figure 1 –
4pi cryogenic microscope: Artist impression of part of the setup. The cryo-stage with the sample is located between the two objectives (air or liquid immersion). The polarized excitation is shown in blue and the STED beams with two orthogonal polarizations in red.
Fluorescent emission, interferometric detection and aberration control are omitted for clarity. Objectives and sample will be inside a vacuum chamber.

Context and state-of-the-art

For more than a century, the law of optical imaging has been that no details smaller than the diffraction limit $\lambda/2NA \sim 200$ nm, with λ the wavelength and *NA* the numerical aperture of the microscope objective lens, can be usefully imaged in the far field. Several methods have been proposed to circumvent the diffraction limit in the last 10-15 years [Klein2014, Schermelleh2012, Huang2010]. Here, super-resolution fluorescence microscopy uses important contributions from single-molecule biophysics and fluorescent protein labelling technology [Moerner2015, Tsien1998], and conceptual proposals by Hell [Hell1994] that date back from as early as the 1990s. The hallmark publications of PALM and STORM in 2006 [Betzig2006, Rust2006] provided the basis for super-resolution microscopy that has become common in today's labs. The Nobel Prize in chemistry was awarded in 2014 to Hell, Betzig, and Moerner for these developments.

There are two main lines of "diffraction-unlimited" microscopy techniques:

1) The first group is based on the concept of **STimulated Emission Depletion** (**STED**) [Klar2000, Hell2009], historically the first technique to image below the diffraction limit in the far field, and the related RESOLFT concept [Hofmann2005]. Traditionally STED is based on a confocal scanning microscope with an additional doughnut-shaped beam, strongly red-shifted w.r.t. the excitation beam, for stimulated emission, restricting fluorescence emission to a narrow spike, smaller than the diffraction limit.

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2) The second group is **Single Molecule Localization Microscopy** (**SMLM**), which is the now generally accepted collective name for variants known by other acronyms (PALM, STORM, fPALM, GSDIM, dSTORM, PAINT, etc. [Hess2006, Foelling2008, Heilemann2008, Sharonov2006, Jungmann2010]). All variants use the concept that fluorescent labels are (photo-chemically) manipulated to switch on and off stochastically, such that at each instant in time only a sparse subset of all fluorophores is in the on-state in which they can fluoresce. Recording many frames (10^3-10^5) of such blinking single fluorophores thus provides a sequence of images of different random subsets of nearly all fluorescent emitters. The active emitters appear as well separated spots that can be identified and processed to provide their

Figure 2 – Single Molecule Localization Microscopy: The complete processing pipeline for generating a 2D super-resolution image based on raw frames from widefield acquisition of sparsely active fluorophores [from Rieger2015].

positions. The localization precision is on the order of $\lambda/NA/\sqrt{n_{ph}} \approx$ 5-30 nm with n_{ph} the number of detected photons (typically a few hundred to a few thousand). Assembling the localization data obtained from all frames (after proper processing, see **Fig. 2**) into one visualization of the final super-resolution image reveals details on the length scale of 10-100 nm, quite a bit higher than the localization precision as the labelling density plays an important role for the resolution too.
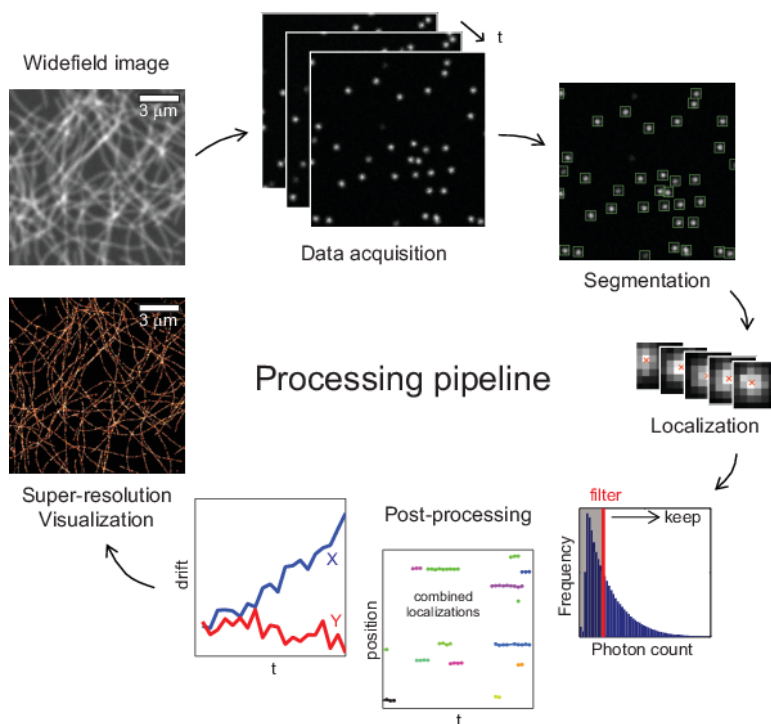
The recent introduction of **Minflux** [Balzarotti2016] is an extension of SMLM where structured illumination in form of a scanned doughnut-shaped excitation beam is used to incorporate extra prior knowledge into the localization process. The beam is scanned in a region of interest (ROI), typically 50 nm. This breakthrough concept can in principle give the same localization precision as with a flat illumination but at about 3-10 lower illumination dose. In its current form the technique is, however, severely limited. First of all, the improvement only holds for emitters that are present inside the tiny ROI, which limits the useful Field Of View (FOV). Second, the tiny ROI must be found within the overall FOV of the microscope with an alternate imaging branch, separate from the branch for photo-collection/detection.

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Limits on resolution & requirements for ultra-resolution

I have introduced the concept of Fourier Ring Correlation (FRC) into super-resolution microscopy for taking all resolution factors into account [Nieuwenhuizen2013]. The FRC quantifies the available image information as a function of spatial frequency, i.e. across all length scales, and finds a resolution limit by a threshold criterion. This resolution measure is now the baseline in the field. The FRC resolution in SMLM can be about 2π times the localization uncertainty if the labelling density is adequate. In general, the best resolution that can be achieved is limited by the total photon count from a single emitter *and* by the density of the emitters labelling the structure [Nieuwenhuizen2013, Legant2016]. Therefore, we need to have high (effective) labelling density *and* high localization precision in all spatial dimensions at the same time for obtaining images with the highest resolution. Below I describe what this means in practice, defining the elements that offer the best solution to the limitations on resolution and that I will bring together in this project.

Localization precision: The localization precision of a single fluorophore scales as $1/\sqrt{n_{ph}}$ with n_{ph} the number of detected photons. Typically a few hundred to a few thousand can be detected per on-state, depending on the type of fluorophore and imaging conditions [Dempsy2011, Avilov2014]. For very specialized imaging conditions higher counts have been reported [Wang2016, Schueder2017], but the highest localization precisions can currently be achieved in **cryogenic fluorescent imaging** [Kaufmann2014, Li2015, Weissenburger2017, Hulleman2018b]. There precisions are in the sub-nanometer range because the fluorescent emitters almost do not photobleach. On the down side, localization based super-resolution is problematic as established protocols for sparsity such as photo-conversion in practice do only work (and poorly so) for a handful of emitters [Chang2014, Weisenburger2017]. Very recently, we presented a first possible solution to this problem using polarization control [Hulleman2018] and consequently, I will opt for this way in this project to achieve the highest localization precision.

3D imaging: Microscopy still often acquires 2D images due to practical limitations of volumetric imaging, such as speed, loss of SNR, photobleaching and poor axial resolution in e.g. confocal microscopy. While light-sheet imaging (or SPIM) [Huisken2004, Legant2016], can image with low light levels and relatively fast, resolutions on the order of nanometers are not possible. STED imaging in 3D [Hell2009b] has been shown with two lenses on opposite sides of the sample (4pi configuration, [Hell1994]), giving an axial resolution in the tens of nanometers. For SMLM, many different solutions for 3D localization have been proposed [Kao1994, Huang2008, Holtzer2009, Pavani2008, Pavani2009, Bourg2015, Descamps2014, Li2018, Franke2017, Mangeol2016, Shechtman2014, Shechtman2015], but all have in common that the axial resolution is 3-5 times poorer than in plane due to decreased signal-to-background and a larger footprint of the point-spread function [Rieger2014], except 4pi interferometric detection [Shtengel2009, Huang2016]. Where the use of two opposing objectives (4pi) doubles the collected photon count [Xu2012, Ram2009, Schmitzbauer2013], only **4pi interferometric detection** has shown the same or even better resolution along the axial direction as in plane. I will therefore build upon this imaging configuration in combination with cryogenic imaging.

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<u>Labelling density</u>: There are a number of emerging developments that address the limitations of labelling technology. Advances in bio-photochemistry have resulted in the development of labels that make a direct covalent bond to the target molecule, such as click-chemistry [Zessin2012], effectively giving a small (<1 nm) label size. Also, techniques to achieve high labelling densities [Legant2016, Strauss2018] have surfaced, but, to date, labelling a structure with sufficient labels is a very hard biochemical problem that often cannot be solved such that a high degree (> 50%) of active labels is achieved [Burgert2015]. The only way to improve the effective labelling density is by combining information, that is, **data fusion**, a technique I have pioneered in the field of super-resolution light microscopy [Loeschberger2012, Broeken2015, Heydarian2018], and that has been applied to elucidate important structural biological questions [Szymborska2013, Engelenburg2014]. Fusion of multiple acquisitions into one reconstruction can mitigate limiting factors of density in cases where many identical copies of the same structure (called "particle") can be imaged. This final reconstruction has effectively many more localizations than each individual SMLM image, which results in a better signal-to-noise-ratio (SNR) and a useful resolution improvement. This idea is conceptually similar to single-particle analysis (SPA) in cryo-electron microscopy (cryo-EM) where image reconstructions have resolutions in the Ångström range while individual particles are barely visible in the raw, noisy acquisitions [Tang2007, Kudrayashev2012]. In summary, I will mitigate the resolution limiting factor due to low labelling density by 3D data fusion.

Benefits of ultra-resolution
There is no general definition of ultra-resolution, but in this project my ambition is to reach resolutions in the small nanometer range in 3D. The key benefit of this resolution range with light microscopy is that it is the *molecular scale*. On this scale, the structure and function of biomolecules organizes life. It is therefore vital to develop enabling tools and methodology as I do here with computational microscopy for imaging. It is also important to mention that my approach can be combined with cryo-EM in one workflow, allowing both structural characterization and functional identification. In the past, super-resolution imaging could for example reveal protein distribution along neurons [Xu2013]. These structures are relatively "large" but required 3D super-resolution with multiple colors. To resolve the composition of macromolecular complexes, another resolution improvement is needed. I envision that through this project we will be able to reveal the 3D distribution of different nucleoporins inside the Nuclear Pore Complex.

Research approach
Below I outline my research approach and objectives for each of the two WPs.

WP1: Prior knowledge and 3D data fusion for 1-nm-scale resolution

Summary: Very recently I achieved an impressive 3.3 nm resolution (wavelength/175) [Heydarian2018] using data fusion in 2D single-molecule localization microscopy with PAINT imaging [Schnitzbauer2017]. In **Fig. 3c**, the central result of this publication is depicted, where 383 individual images of a very efficiently labelled (80%) artificial DNA-origami nano-structure, in the form of the TU Delft logo, show details below 4 nm (collaboration with Jungmann, LMU Munich [Jungmann2010]). I will build upon this breakthrough framework developed in my ERC Consolidator Grant and take the

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fundamental step from 2D to 3D. Next to the more involved acquisition in 3D, the required labelling density to align particles robustly in 3D is expected to be higher than in 2D as we have to deal with more rotational and translational degrees of freedom in the registration (parameters in x,y,z and three angles compared to x,y and one in-plane angle only for 2D). I propose to:

WP1.1: Extend the cost function to include the (potentially a-priori known) **symmetry** of the particles to restrict the search space for the optimization;

WP1.2: Include **photophysical models** derived directly from the localization data to be less sensitive to repeated localizations of the same molecule;

WP1.3: Identify **biological heterogeneity** using maximum likelihood estimation (MLE)-based and iterative multivariate statistical analysis (MSA) schemes;

WP1.4: Introduce automated instead of manual **particle picking** in 3D based on machine learning approaches via convolutional neural networks similar to Chen2017.

Rationale: Traditional methods for fusing multiple SMLM images of a single underlying structure can improve SNR and resolution, but use templates or are very sensitivity to initial registration errors. These approaches typically copy directly algorithms from cryo-TEM data fusion or Single Particle Averaging (SPA), whereas the physical image formation in cryo-EM and in SMLM is completely different. In cryo-EM, the interaction of the electrons with the electrostatic potential of the specimen is imaged on the atomic scale, whereas in SMLM, single fluorescent molecules labeling the structure of interest are localized. The imaged particles in cryo-EM are truly the same in all acquisitions while noise is randomly distributed. The noise is averaged out after alignment, increasing the SNR with each added particle. In contrast, in SMLM, not the structure itself is imaged but the positions of the fluorophores attached via a linker to the underlying structure. Furthermore, in practice the labeling is often incomplete where a 30-70% density of labeling (DOL) is typical [Burger2015]. In addition, statistical variations in localization uncertainty, false positive localizations [Linde2010, Sinko2014, Fox-Roberts2017] and the statistical distribution of repeated localizations of the same fluorophore are additional sources of randomness that make the image data more complex than in cryo-EM. A
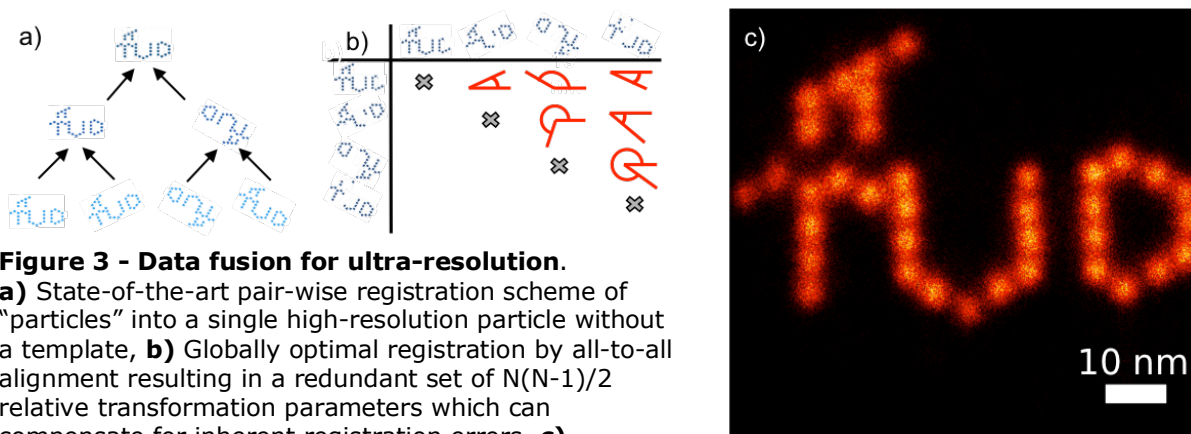

Figure 3 - Data fusion for ultra-resolution.
a) State-of-the-art pair-wise registration scheme of "particles" into a single high-resolution particle without a template, **b)** Globally optimal registration by all-to-all alignment resulting in a redundant set of N(N-1)/2 relative transformation parameters which can compensate for inherent registration errors, **c)** Reconstruction result from 383 individual super-resolution 2D PAINT images of a DNA-origami structure obtaining wavelength/175 resolution [Heydarian2018].

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number of studies have applied SPA algorithms from the cryo-EM field to SMLM despite these fundamental differences [Engelenburg2014, Gray2017, Schnitzbauer2017, Salas2017]. All these approaches first convert localization data to pixelated images and then apply class-averaging algorithms, making it impossible to take into account the mentioned differences of the point datasets that make up a SMLM image. I have pioneered data fusion in SMLM by using an 8-fold symmetric ring as a template to align nuclear pore complex (NPC) images [Loeschberger2012]. The problem of a template is, however, to generate a structure that is biased towards this template. In the EM community, this problem has been addressed extensively [Henderson2013], and a priori templates are now abandoned. A template-free approach for SMLM datasets based on a pyramid registration procedure has been proposed by us before, cf. **Fig. 3a** [Broeken2015]. Unfortunately, this method, as any iterative method of combining pairwise registrations, suffers from a large sensitivity to registration errors in the bottom layer of the pyramid, which propagate into subsequent layers of the procedure.

The above problems have inspired me to start investigating a global method to align *N* particles, taking into account the maximum information from registering each particle to all others, creating redundancy for improved robustness (see **Fig. 3b**). Each pair registration results in
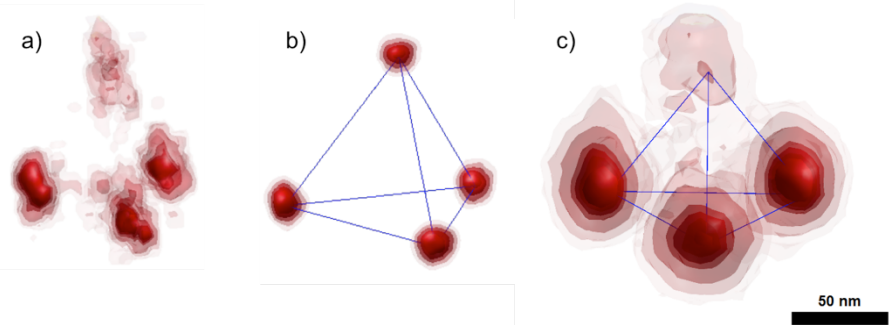

Figure 4 – 3D Data fusion.
a) Example of a single tetrahedron imaged with DNA-PAINT, **b)** 3D reconstruction *with* a template from 499 pyramids [Feilzer2015], **c)** Preliminary 3D reconstruction *without* a template [unpublished].

the best estimate of the relative orientation and position of the two particles. What is needed, however, are the absolute orientations and positions of all particles. We solved this by Lie-algebraic averaging, inspired by the computer-vision field *structure from motion* [Govindu2004, Govindu2014, Ye2017]. The individual registration is done by optimizing a variant of the Bhattacharya cost function [Broeken2015, Heydarian2018] (similar to a Gaussian with the distance between two points/localizations in the argument), which can take into account possible anisotropic localization uncertainties. This cost function and the all-to-all optimization are computationally expensive as the latter needs $N(N-1)/2$ pair-registrations ($N{\sim}100$-5000) and the former scales as the square of the number of localizations per particle. Clever algorithms and pre-alignments with Gaussian mixture models [Jian2011] restrict the parameter search space. In addition, GPU implementations have been important in our 2D effort to accelerate computations, and will therefore be key to take this work to 3D. Bringing in prior knowledge helps tremendously for registration, e.g. in Feilzer2015 we have applied a tetrahedron model for 3D fusion of DNA-Origami nano-structures, see **Fig 4a,b**. With our new global approach we could generate a preliminary reconstruction, albeit worse than with template, as shown in **Fig. 4c**.

From the above discussion I conclude that it is necessary to work on the following topics to realize 3D data fusion for ultra-resolution:

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WP1.1: Symmetry: An incomplete density of labelling (DOL) is very detrimental to the quality of the registration. In my experience, the registration usually works fine down to a certain DOL, and breaks down completely after dropping below a threshold [Heydarian2018]. The exact value is found only empirically and depends on the number of binding sites on a particle but also on the particle's shape (irregular vs symmetrical). From the biochemical sample preparation side, it is often very difficult to improve the protocol such that more sites are labelled. The best option to reduce the requirement on the DOL is the inclusion of prior knowledge in the form of symmetry, as many biomolecules are (partially) symmetric due to the repetition of subgroups/units. For example, tobacco mosaic virus is a foremost showcase of such symmetry studied in EM.

In WP1.1, I aim to include symmetry into the cost function to restrict the search space. The approach in the EM software cannot be transferred to 3D SMLM data as EM software groups the projections into projection classes for the different angles and once the angles are assigned the symmetry can be applied. Currently Lie-algebraic averaging and quaternion representations are used to obtain a continuous representation of the rotation group, which allows for much easier numerical optimization of the cost function. Conceptually, this can be transferred to 3D, but inclusion of symmetries is very challenging as e.g. the orientation of the assumed symmetry axes is unknown a priori and cannot straightforwardly be included by another rotation group representation in the cost function optimization.

Another way to tackle this difficult problem is to randomly redistributed the localization data while obeying the imposed symmetry, but this requires again that the orientation of the symmetry axes must be found. I will also explore a different approach, in which I will test to what extent symmetry is present in the data. This requires the development of new methods as the localizations represent point clouds and they do not show any symmetry in the strict mathematical sense. Instead they are only roughly symmetric, subject to the different noise sources in SMLM datasets mentioned earlier. I will start by investigating ideas from image processing such as scale spaces.

WP1.2: Photophysical models: The distribution of the number of localizations over the different binding sites is never uniform, as there are variations in labelling efficiency, but also because of statistical variations in the number of independent localization events originating from the same fluorophore. The distribution of these statistical variations depends on the type of imaging modality (e.g. STORM, PALM or PAINT type) due to differences in the photo-bleaching mechanism [Annibale2011, Grussmayer2014, Nieuwenhuizen2015]. This can result for e.g. STORM in a skewed distribution of the number of localizations per binding site. The consequence of these statistical variations for the particle registration process is that binding sites from different particles that by chance have more localizations than other binding sites tend to align. This creates an accumulation of localizations for one binding site, yielding a "hot spot" artefact in the reconstruction [Heydarian2018]. As a way out, I will explore the possibility to include the probability distribution of localizations per binding site into the registration as a prior, by using a maximum a posteriori (MAP) optimization, similar to Bayesian approaches in SMLM [Cox2012]. The switching kinetics parameters can be obtained from the localization data itself [Nieuwenhuizen2015, Annibale2011] and can subsequently be included in an analytic model for the registration prior. Another option is to cluster the localizations within each particle prior to registration or to down weigh regions of very high

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localization density. Cluster identification is a very active field [Veatch2011, Rubin2015], in which very recently the Renyi divergence seems to have solved problems of over and undercounting [Staszowska2018], implying that I can follow a number of different approaches to achieve my goal. I can even use computationally very expensive concepts as I only have to deal with the localizations of one particle at a time.

Another positive impact, besides a mitigation of the "hot spot" problem, is a more favourable scaling of the FRC resolution measure as a function of the number of particles. This could reduce the number of required particles to achieve a certain resolution, or increase the resolution at a given number of particles even further. The FRC resolution as a function of the number of particles scales in cryo-TEM SPA as expected with the square-root of the number of included particles [Voortman2014], but interestingly, so far this scaling is observed to be worse in SMLM [unpublished]. This discrepancy stems from the fact that the particles in SMLM are not truly identical because different (subsets) of fluorescent labels are imaged and their positions are randomly distributed within the localization uncertainty.

WP1.3: Biological heterogeneity: My goal here is to identify biological heterogeneity, that is, multi-class classification from the data where either multiple different macro-molecular structures are present or variants. Variants can consist of addition/removal of other small sub-groups to/from the macromolecules. In most EM software, k-means clustering [Tang2007] is still applied with user-defined k classes in combination with multi-reference alignment and/or multivariate statistics [Heel1985] and subsequent hierarchical classification. For example, the NPC can deviate from the mostly seen 8-fold symmetry and exhibit 9 blobs [Loeschberger2014]; however, in a data set both fractions occur and automated data fusion could therefore hinder the discovery of new biology because also the small fraction of 9-fold NPCs would be "averaged" into the dominant class of 8-fold NPCs.

I will start by investigating if ideas from [Hadani2011] can be transferred to 3D as these authors already use a Lie-algebra averaging framework in the classification process. However, I also anticipate that establishing a new method for multi-class identification would best be evaluated on 2D simulated and experimental data of known (nano)-structures and then transferred to 3D data.

WP1.4: Automated particle picking: For data fusion in SMLM, currently only a few hundred particles have been combined [Loeschberger2012, Symbroska2013, Broeken2015, Sala2017, Heydarian2018], as opposed to cryo-EM, where 10^4-10^5 particles are not uncommon [Bartesaghi2012, Schur2013]. Clearly, adding more and more particles will improve SNR also for SMLM. The drawback is that finding these particles in the acquired images by handpicking will take more and more time (from one afternoon to several days). The standard practice in EM for optimal reconstruction is still handpicking, as the SNR in raw projections is close to zero and robust automatization is extremely hard. Inclusion of dirt, noise or other fragments evidently impairs the quality of the reconstruction. Very recently, the use of convolutional neural networks, so-called "deep learning" [Chen2017], has been successfully demonstrated to learn the picking after training from handpicked particles. No cryo-TEM methods exist that can directly be applied to 3D SMLM data; I will start by applying a maximum projection to the 3D data onto the xy-plane as little overlap in 3D between particles is to be expected from the

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acquisition (the volume is much wider and deeper than high). This will increase the SNR and make manual inspection much easier. I have already tried a few automated approaches already, but this did not result in a robust detection of particles [unpublished] and will therefore investigate machine-learning approaches too. A human operator can easily and fast pick a few tens of candidates from 2D projections as the training set. The time gain and reliability improvement for this routine task at the start of each reconstruction is therefore a highly desirable feature.

All the above topics need efficient computation. I expect that a substantial amount of time will be spent on implementation of the algorithms for parallelization on GPU cards, as unfeasible computational times of days or weeks would seriously hamper the project.

WP2. 3D isotropic imaging on the 1-nm scale with a 4pi cryogenic setup

Summary: Cryogenic imaging offers sub-nanometer localization precision because of the virtually unlimited photon count [Li2015, Weisenburg2017, Hulleman2018b] but lacks ways for on-off switching of fluorophores [Weisenburger2017]. Within my current ERC Consolidator Grant we have presented a solution to obtain on-off switching for fluorophores in a frozen/cryogenic environment using orientational STED based sparsity, cf. **Fig. 5b** [Hulleman2018]. Enabling on-off switching is the key requirement to obtain single-molecule-based diffraction unlimited imaging by sparsity [Hell2007, Hell2009]. Now I propose a major step forward by:

WP2.1: Adding a second opposing objective lens and replacing the large, bulky liquid-nitrogen tank with a small cryo-stage to cool the sample, which allows imaging from opposite sides, a so-called **4pi cryogenic** setup;

WP2.2: Enabling **3D polarization controlled STED**, which will give better sparsity and is key to higher resolutions. Additionally, the 4pi-setup collects twice the number of photons, but most importantly offers a ~10x better axial resolution by interferometric detection;

WP2.3: Add **aberration control** to both emission arms to maintain ultra-resolution over an extended depth range (1-10 μm). Here we will start to image nucleoporins inside the NPC, but also other biomolecular complexes from our collaborators.

Rationale: Traditional switching mechanisms for on-off switching of emitters do not work at cryogenic temperatures as they require a conformational change of the fluorophore itself or a liquid solution for chemical interaction with the fluorophores. At low temperatures and in a frozen state both options are very inefficient [Chang2014, Kaufman2014, Weisenburger2017]. In order to have the benefits of high photon counts, long term stability and the gentle sample fixation of cryogenic imaging we have introduced polarization controlled orientational STED-based sparsity [Hulleman2018]. We use a linearly polarized excitation beam that is rotating (inspired by Hafi2014, but with proper polarization on the sample for deep modulation) to selectively excite the fixed dipoles. The excitation efficiency scales as $\cos\alpha^2$ with α the angle between the (linear) excitation polarization and the dipole axis. This results in an on/off ratio of unity, which is insufficient. Adding a STED beam with orthogonal polarization to the excitation beam (cf.

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Fig. 5b) we find that the angular emission profile after depletion has a full width at half maximum (FWHM) of $\frac{\pi}{2}/\sqrt{1+\frac{I_{dep}}{I_0}}$, with I_{dep} the depletion intensity and I_0 a constant given by the fluorophore properties, allowing for FWHM narrowing as in standard STED (using reasoning along the lines of Hell2007). Replacing the "standard" large, bulky liquid-nitrogen tank [Li2015] with a small cryo-stage to cool the sample allows imaging from opposite sides (see **Fig. 1** and **Fig. 5a,c**). This will enable much better STED polarization control in 3D and thus better sparsity and resolution. In addition the 4pi-setup collects twice the number of photons, but most importantly offers a ~10x better axial resolution by interferometric detection [Shtengel2009, Engelenburg2014, Huang2016], compare **Fig. 5c**. I anticipate that double aberration correction (**Fig. 5c**) is needed [Huang2016] together with fitting of experimentally retrieved points-spread-functions (PSF) for localizing the fixed dipoles [Stallinga2012, Backlund2012, Lew2014], or novel deep learning techniques [Nehme2018]. For the calibration we need to move from simple "guide star" solutions to calibration and phase diversity protocols [Hanser2004, Wilding2018] tailored to cope with the fixed emission dipoles of the fluorophores.

From the above discussion I conclude that it is necessary to work on the following topics for realizing an experimental setup for ultra-resolution:

WP2.1: 4pi cryo: In the artist impression in **Fig. 1** and the schematic sketch of **Fig. 5a** the cooled sample holder will be small (about 1x1 cm). The cooling will be SQUID-based and provided by Demcon-Kryoz. Delmic will add it onto a stable stage for us. The objectives will be placed inside the vacuum chamber but at room temperature and we will add heat shields between the objectives and the sample. We will plan the design of the vacuum chamber and cooling stage such that that cryo-transfer will be possible for subsequent cryo-EM imaging at the end of the project. We will start with fixated samples to establish the protocols [Kopek2017]. As imaging times will be likely several hours, we need i) a very stable stage (drift <5 nm/min) especially in axial direction, ii) a very stable temperature in the lab (<0.1 K), and iii) drift correction during the experiment and iterative marker based post-processing drift correction [Mlodzianoski2011, Geisler2012, Dai2016]. Initially we will use long-working distance air objectives as nearly all features of the setup can be realized with lower NA ~0.7, except the out-of-plane polarization control. Later we can switch to 0.9 NA air objectives, and the final solution will be to
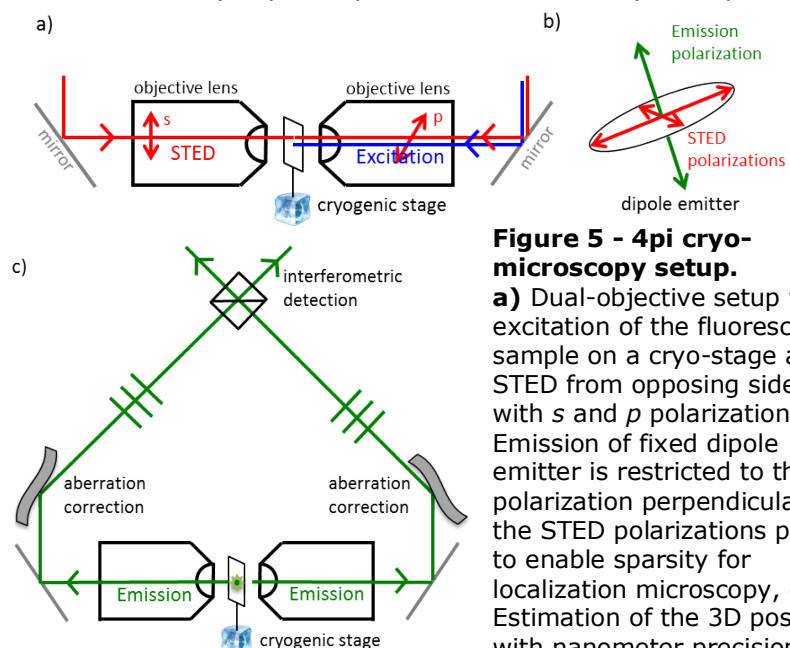

Figure 5 - 4pi cryo-microscopy setup.
a) Dual-objective setup with excitation of the fluorescent sample on a cryo-stage and STED from opposing sides with *s* and *p* polarization, **b)** Emission of fixed dipole emitter is restricted to the polarization perpendicular to the STED polarizations plane to enable sparsity for localization microscopy, **c)** Estimation of the 3D position with nanometer precision with aberration-corrected interferometric detection.

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use immersion at cryogenic temperatures. Then we need to bridge a steep temperature gradient as we want to keep the objectives at room temperature. Either we will use solid immersion (SIL) or a design with liquid immersion as recently proposed [Faoro2018, Fuest2018]. The setup will be designed for imaging with two colours depending on the evaluation of the fluorophores [Raulf2014, Verja2015, Dempsey2011, Grimm2015].

WP2.2: 3D polarization controlled STED: We will build up the complexity of this complicated setup step-by-step. Several beams of different polarization need to work together while the polarizations on the sample must be as desired (cf. **Fig. 5b**). In Hulleman2018 we have shown how to ensure proper polarization while the beam passes through several imperfect optical elements. Once this calibration is established, we can start with widefield polarized excitation and non-interfering 4pi detection, followed by STED from one and then two sides, and finally by two-objective interference detection. For ease of experimenting we will start with samples embedded in a gel/matrix at room temperature. The main goal of full 3D 4pi polarization control also requires out-of-plane excitation and depletion polarizations for isotropic imaging, otherwise we "miss" molecules with dipole orientations tilted with respect to the sample plane. We can then first localize single emitters in 2D in the 4pi configuration without interferometric detection, but estimate their 3D emission dipole orientation [Backlund2012, Stallinga2012] by investigating the different polarization channels. With an spatial light modulator (SLM) present in both emission arms we could investigate standard 3D imaging techniques using astigmatism or defocus [Kao1994, Huang2008, Holtzer2009], but only the interferometric detection will yield isotropic resolution. To this end we must develop new ways of fitting the spots as the emission patterns of the fixed dipoles will not be round Gaussian shaped. I envision that we can bring our experience with the use of full vectorial PSF fitting, in combination with computational efficiency, to the table to solve this challenge [Smith2010, Rieger2010, Stallinga2012, Broeken2014, Smith2015b].

WP2.3: Aberration control: We plan to place SLMs in both emission arms of the setup, see **Fig. 5c**, as in Huang2016 in their localization based 3D setup. For the SLM we can use either reflective Liquid crystal on silicon (LCoS) as we and others have used before [Broeken2015, Thorsen2018, Wilding2018] or deformable mirrors [Gustavsson2018, Huang2016, Burke2015, Tehrani2015]. In our case of widefield STED it is less critical to correct the excitation than for standard donut STED where the resolution depends very critically on that the center of the donut is zero [Hell2007, Goud2012, Kromann2012]. The aberration calibration step cannot use a simple "guide star" protocol for the full 4pi setup as even the image of a point source, a single emitter, is reimaged depending on its axial position and dipole orientation. Phase diversity protocols [Hanser2004, Wilding2018], but tailored to cope with the fixed emission dipoles of the fluorophores will be develop to this end.

In WP2.3, together with our collaborators (Sauer, Jungmann, Grünwald), we will also investigate the effect of labelling on the apparent size of structures, i.e. the influence of the fluorescent labels on resolution as it is known that this can have an effect [Ries2012]. A number of control experiments with different known structures, e.g. microtubule or DNA nanostructures should clear up to what degree this effect is present. Later we will move to imaging the NPC from our collaborators (Jungmann, Grünwald). They will decide which nucleoporins to image as the labelling will play a crucial role here.

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2a2. Research plan

Work plan and time line

The research is subdivided into two work packages (WPs): WP1 **3D data fusion** and WP2 **4pi cryo**. The table below outlies the project planning and allocation of the different resources to the different tasks in the work packages. The image processing team (PhD1 and PhD2) will interact with the experimental team (PhD3, PD and technician) to collectively enable the breakthrough in structural imaging on the 1-nm scale. The table is indicative and will be adjusted during the project if needed.
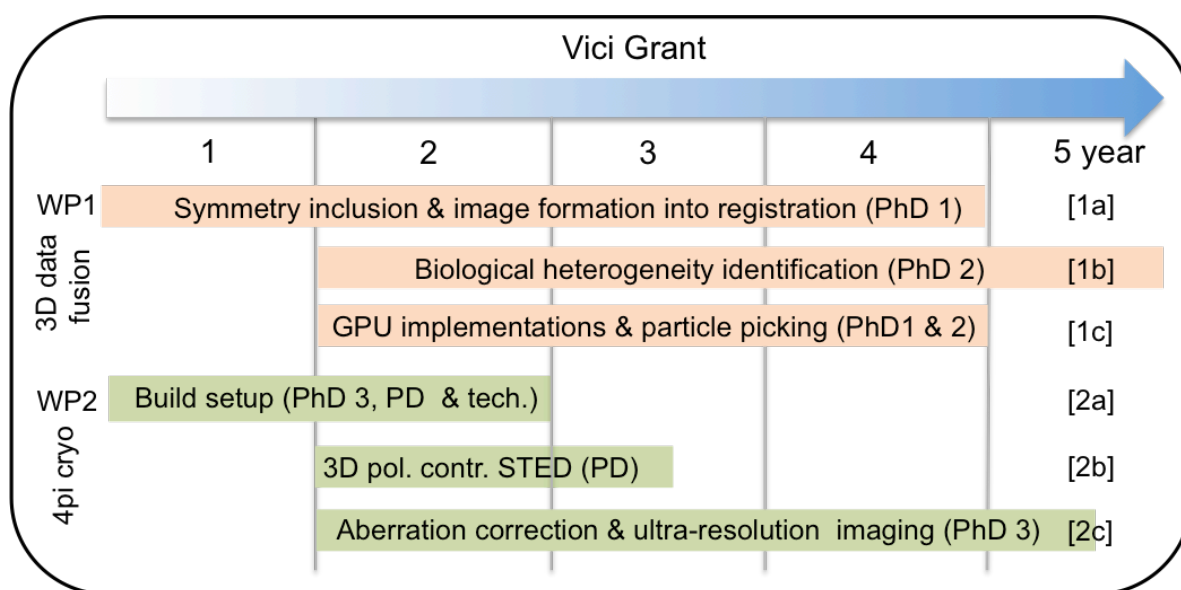

Table 1: Planned timeline over the project duration of 5 years.

WP1: 3D data fusion

Task 1a: We will build upon the use of the Bhattacharya cost function and Lie-algebraic averaging introduced by me [Heydarian2018] in 2D to align particles for 3D. Here, the much larger search space needs to be tackled in particular. Furthermore, we will include **image formation** models to explicitly handle several aspects intrinsically related to SMLM [Nieuwenhuizen2015b] compared to cryo-TEM SPA: incomplete labelling and repeated localization of the same emitter to avoid the "hot spot" problem in the registration. In a next step we will impose potential **symmetries** of the particles directly into the cost function which will help tremendously to be able to align data. Additionally, we will develop measures for how symmetric a particle is when not imposing additional prior knowledge.

Task 1b: In order to be able to identify **biological heterogeneity** or drug target sites in the data during registration, we will develop automatic multi-class identification of a-priori unknown different classes (= variants of the same overall structures) of particles within one data set. I envision using a combination of iterative multivariate statistical analysis and multi-reference alignment followed by hierarchical classification to determine the discrete classes.

Task 1c: We will realize robust **automated particle picking** in 3D. This is a practically important issue and potentially very time-consuming as in 3D no easy solutions

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exist in cryo-TEM SPA. Additionally, we will develop code optimized for speed on **GPU** as I still anticipate computation times that could impair practical applicability during Task 2c (<1h for a first reconstruction; <1d for a full reconstruction).

WP2: 4pi cryo

Task 2a: We will build upon my and my group's strong experience with cryogenic setups [Hulleman2018, Hulleman2018b], but now develop a **4pi cryogenic** ultra-resolution fluorescence microscope. The technician will assist the PostDoc and PhD3 in the construction of custom-designed parts for the vacuum chamber and 4pi optics, and electronics for instrument automatization. In particular, we need a very stable feedback stage for long imaging times with as little drift as possible. The setup will be extensively evaluated, initially on artificial structures (DNA origami at room temperature) in terms of 2D and 3D resolution, but later (on long-term imaging stability) under cryogenic conditions. First we will use air objectives in the setup, followed by (fluid cryo) immersion to access the 3D polarization state of the emission and obtain higher photon counts.

Task 2b: We will demonstrate single-molecule-based localization 3D microscopy in frozen samples by **3D polarization control STED**. We will employ a step-by-step approach to get each aspect of the imaging right as the many different beams of different polarization need to work together, i.e. starting with widefield polarized excitation and non-interfering 4pi detection, followed by STED from one and then two objectives, and finally by two-objective interference detection. We will start by fixing (the emission dipoles of) single molecules at room temperature in a matrix/gel for faster evaluation and later at cryogenic conditions. This task relies, of course, on the realization of the setup in Task 2a.

Task 2c: We will realize aberration-free imaging by adding **aberration control** optics into both emission arms of the 4pi microscope in order to increase the axial and in-plane resolution towards 1 nm. This allows imaging into whole cell samples (~10 μm) while maintaining the ultra-high resolution. Imaging of different sample such as the different Nups of the NPC will be a larger part of the task. New ways to realize the wavefront sensing will be developed as guide star solutions cannot cope with the fixed emission dipoles of the fluorescent labels. The goals of this Task rely on *all the other* tasks, including those from WP1. Therefore, we will image structures as early as the setup can generate images to provide feedback to the other Tasks.

My group has leading expertise in algorithm development and (cryo) super-resolution imaging. I will devote 40% of my time to this project and assemble a dedicated team consisting of **3 PhD** students (4y each), a **PostDoc** (2y) and a **technician** (50% for 2y). The broad and interdisciplinary nature of the proposed research will be challenging. My group is already interdisciplinary, comprising PhD students and PostDocs with backgrounds in physics, electrical engineering and computer vision. The individual skills and interests of each newly hired researcher must match the requirements of the individual sub-project, while strengthening the team as a whole at the same time. For the Vici project, I will hire **PhD1** and **PhD2** (WP1) with a background in electrical engineering and/or computer vision with an interest in statistical analysis and symmetry groups. At least one of them should have a high affinity with computing and GPU

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programming. In the experimental research line (WP2), the **technician** with a background in machining and construction will assist in building the setup. PhD3 and the PostDoc should be strong experimental physicists. The **PostDoc** should have expertise in light microscopy, ideally recruited from a (collaborator) lab where he/she has already worked with or even built a 4pi microscope. **PhD3** preferably has a background in biophysics to facilitate easier interaction with the collaboration partners.

Infrastructure

The experimental work will be carried out at the Van Leeuwenhoek Laboratory (VLLAIR). This Center of Excellence of Delft University of Technology, run by my department (Department of Imaging Physics within the faculty of Applied Sciences), offers suitable low-vibration (<class VC-C), high-thermal-stability (<0.1 K) lab space and several biological, chemical, and biochemical labs for sample preparation and cell culture. All is located within the same building where my research group is situated. Cryo-electron microscopy facilities, including plunge freezers, are available on-campus at TU Delft's Department of Bionanoscience, with whom I frequently collaborate. In terms of computational infrastructure, we do not need computation on the level of national super computers but smaller scale GPU and CPU servers. Currently we have 3 older 48-core CPU servers and 4 Tesla K40 GPU cards. In this project we apply for a dedicated GPU server to successfully run the project. Having decent computation power is needed for testing and experimenting; even so the final computations should run on a single GPU desktop in a lab within a few hours.

Collaborations

In this project, we will collaborate with leading international biophysics/technology university groups to complement our research expertise but also to apply our technology. The collaborations cover the wide range of expertise areas involved. Several visits of team members to the different collaborators are expected, which can be short visits, but also extended stays of up to two months.

- Ralf Jungmann (LMU München, Germany) who is leading in DNA PAINT-based imaging and DNA nanotechnology. His group previously created the TU Delft logo from DNA-Origami shown in **Fig. 4c** [Heydarian2018]. His nanostructures will be used as reference structures to evaluate and improve the performance of the algorithms in WP1 on room-temperature acquisitions. In addition, he is keen to apply our algorithms directly in his lab during acquisition.
- David Grünwald (U. Massachusetts at Worcester, USA) is a leader in imaging single molecules inside the cell, in particular mRNA transport through the nuclear pore complex. We have an ongoing collaboration on cryo light microscopy. Grünwald will support the Vici project in terms of sample preparation know-how.
- Markus Sauer (Würzburg, Germany) is recognized as pioneer in designing, understanding and applying fluorescent labels in super-resolution microscopy. He will contribute his knowledge about photophysics of dyes to our work on cryogenic and STED imaging modes.
- Mark Bates (Göttingen, Germany) is one of the inventors of STORM [Rust2006] and has in-depth understanding of photophysics of dyes and the different flavours of 4pi microscope setups. He is willing to transfer his know-how to us by hosting our team (PostDoc/PhD3/technician) for two months.

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- <u>Jörg Bewersdorf</u> (Yale, USA) is willing to share his aberration correction and 4pi imaging [Huang2016], as well as his STED know-how with us.

Within the Netherlands, collaborations include users from the biology field such as Peter Peters & Raimond Ravelli (Maastricht University; correlative cryo LM-TEM & supply of samples) and Kees Jalink (NKI Amsterdam; supply of samples). Locally, at TU Delft, my key research partner Sjoerd Stallinga is bringing long-standing expertise in optical theory and optical design to the table. For aberration and system control expertise, Michel Verhagen & Carlas Smith will be valuable sources of feedback and inspiration. TU Delft also offers a strong EM community including Jacob Hoogenboom and Arjen Jacobi (cryo sections). Hoogenboom is leading an initiative to integrate several steps in the cryo-EM sample preparation workflow into one apparatus, which reduces the number of cryo-transfer steps, thereby decreasing sample preparation time and increasing reliability. Our platform for the cryo-LM stage will be designed by the same company, Delmic, as in Hoogenboom's initiative. In this way, our platform has the potential to be seamlessly integrated with sub-nanometer cryo-TEM imaging.

Risk assessment

An ambitious project such as this one is not without risks. The following risks can be identified:

Construction of an **ultra-stable cryo-stage** with <5 nm/min drift is a very challenging task, but I have found commercial partners that together can deliver such a stage. Detailed COMSOL simulations conducted by DEMCON-Kryoz in the preparatory phase of this proposal have indicated that the microcooling system can even reach 1 nm/min stability. In case it is significantly delayed or unstable, we will fall back on room-temperature imaging with suboptimal caged-dyes or PAINT imaging. The data fusion will still give results albeit at a lower resolution (probably ~5 nm range).

Construction of the **4pi microscope** itself will be a challenge. Only a handful of groups in the world have built a 4pi setup, but none with a cryogenic sample nor with polarization-controlled emission and STED. In addition, all commercial systems so far have not been widely adopted in the community. I foresee in particular the stable alignment of the different beams with respect to each other and the polarization control over several mirrors to be critical. Next to our excellent local machine shop that can build required custom components for this very advanced microscope, we are able to transfer setup know-how from Mark Bates (who has built several versions of 4pi setups) to our team. He is willing to host our entire experimental team if needed to realize our setup.

From the computational side I see potential risks albeit on a smaller scale. The most impractical feature of complex algorithms for this project is that of unpractical **computational times**. These could result from the scaling behavior of the envisioned algorithm for the all-to-all registration (scales as N^2). This impacts the ability to test and experiment with different algorithms and run a large-parameter-space simulation. In the end, computations longer than 1 day for the registration of one data set will hamper dissemination to end-users in biological labs. We expect that gaming GPUs (<$1000) will be powerful enough if we parallelize the algorithms (as we have done in the past). Another potential problem could be **"low-quality" experimental data**, i.e. data that is corrupted with residual drift and/or has too bad localization uncertainty to test the algorithms to their full potential. In that case we must revert to simulation data.

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2b. Knowledge utilisation

As an enabling tool, the outcomes of this project will impact the biomedical community as well as provide possibilities for commercialisation by the microscopy industry. The biomedical field would benefit from seeing the structure *and* protein composition of e.g. (macro)molecular assemblies in 3D at nanometer resolution. Where recent advances in cryo-TEM allow to see structural detail in the sub-nanometer range, my technique has the spatial resolution in fluorescence microscopy to also resolve protein identities. In short this combination has the prospect to observe interacting proteins, viruses and pathogens in their native environment for healthy and diseased cells to understand molecular mechanism of physiology. In the long run that should help to cure diseases by studying the interaction of targeted drugs with specific proteins.

Promoting knowledge utilisation is therefore along three lines: (1) Disseminating the outcomes through scientific articles and conference contributions, (2) Commercialising cryo-stage microscopy hardware, and (3) Making available open-source software for the data fusion reconstructions.

On the **hardware** side I will collaborate closely with Delmic BV as commercial partner as they build and market correlative light-electron microscopes and currently have a larger initiative to develop a cryo-stage for their market. I will also collaborate with Demcon-Kryoz, who will supply the (SQUID-based) micro-cooler used in the proposed technology. I have been actively involved in starting the collaboration between Delmic and Demcon-Kryoz. During the development and implementation phase of my cryo-stage, I plan bi-weekly meetings with Delmic to align my design efforts with those by Delmic for correlative microscopy. In this way, my techniques will be directly transferrable to correlative light and electron microscopy applications. I anticipate that the design and later implementation phase will be completed after the first year of the project. Delmic recently formed a consortium with several universities (including the group of Jacob Hoogenboom at TU Delft) and research institutes (USA, UK, Germany) to design and build new integrated cryo-FIB-SEMs to facilitate sample preparation for high-resolution cryo-TEM. Therefore, my collaboration with Delmic provides direct access to an international emerging market. E.g. within the Dutch National Roadmap for Large-Scale Scientific Infrastructure (NEMI) alone 8M€ have been allocated for cryo-TEM and correlative cryo electron-light microscope hardware.

The project will be carried out in the Van Leeuwenhoek Laboratory for Advanced Imaging Research (VLLAIR), a Center of Excellence of Delft University of Technology, run by my department. In this facility we assemble our latest innovations in different branches of imaging technology and work with end users and industrial partners to maximize our societal impact. My colleagues Jacob Hoogenboom and Pieter Kruit also utilise VLLAIR to interact with their industrial partners and potential users. This will result in synergy in terms of executing the Vici project and in opportunities to showcase our innovations to a wider network of potential users.

With David Grünwald (University of Massachusetts Medical School, USA) I currently have a collaboratively funded NIH project that in particular involves development of a (standard) cryogenic light microscope for studying chromosomal structure. He has expressed his strong interest in using the proposed hardware for higher-resolution 3D imaging.

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On the **software** side I will distribute the new algorithms open-source, such that other *developers* (not biological end-users) can use our findings freely and without time loss to recode our algorithms. For the dissemination I will continue to use our image processing library DIPimage (www.diplib.org). I have been one of its principal architects and now, as scientific director of the project, initiated a recoding from scratch of the 20-years-old code. For end users in the biology community I will additionally provide ImageJ plugins for maximal impact. These efforts constitute a significant amount of work (commenting, cross-platform and version testing but foremost maintenance), but in my experience it is rewarding not only as service to the community but also as starting point for new collaborations later on.

For this project, in particular computation times are important as the algorithms can take days to run on simple PCs. Therefore, next to standard CPU code, I will also provide GPU code, which makes use of the multi-core architecture of graphics cards. Nowadays even cheap GPU cards (~$500 - $1000) fitted in standard PCs are capable of speeding up algorithms suitable for parallelization more than 100x. As my algorithms are suited for parallelization, I can obtain fast results in the labs of biological end-users without the need of dedicated servers. I pioneered this idea for localization of single molecules without compromising on the algorithmic quality [Smith2010], when GPU programming was still a niche. For our current algorithms, next to standard CPU code, we have made GPU code available in the form of C-code and Matlab scripts (from github) [Heydarian2018], using funding from the Dutch eScience center in Amsterdam (www.esciencecenter.nl). The GPU implementation is crucial for the translation to the use labs of the all-to-all registration pipeline for localization microscopy data. Otherwise end-users need dedicated servers with runtimes of 1-2 days, which would be prohibitive in the dissemination.

I will use the new software on data from within my network in the Netherlands, including Erik Manders (University of Amsterdam), Adriaan Houtsmuller (Erasmus MC, Rotterdam), Kees Jalink (Netherlands Cancer Institute, Amsterdam) and locally at TU Delft, which will demonstrate applicability for a wide variety of use cases and help dissemination to a wide research community.

My track record in transferring software to academic and industrial partners includes work on defocus estimation [Vulovic2012], which has been transferred to FEI (now ThermoFisher) and is now integrated in their EPU TEM release software. On the end-user side, early involvement in algorithm development resulted in a number of co-publications where the software could be directly coupled to biological questions [Loeschberger2012, Smith2015, Nieuwenhuizen2015].

Users have already agreed to participate in the **user committee** which will meet twice a year on-site. Covering all sub-fields relevant to this interdisciplinary project, the committee consists of: Peter Drent (CEO confocal.nl), Sander den Hoedt (CEO Delmic BV), Raimond Ravelli (correlative light and electron microscopy, Maastricht University), Lukas Kapitein (cellular biophysics, Utrecht University) and Erik Meijering (biomedical image processing, Erasmus MC).

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2c. Number of words used

section 2a: 7459   (max. 8,000 words on 16 pages)

section 2b: 1000   (max. 1,000 words on 2 pages)

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2e. Data management

1.   Will data be collected or generated that are suitable for reuse?

The data that will be handled in this project can be subdivided in two categories:

-   i)   Simulation image data, used to demonstrate and validate the performance of data fusion algorithms. These data will be made available with the publications resulting from this project, along with the source code.
-   ii)   Experimental super-resolution images, these are saved on our microscopy server, transferred to internal TU Delft (raid)servers subject to daily backups.

2.   Where will the data be stored during the research?

All simulation data will be stored on internal TU Delft servers and will be transferred to a publicly available ftp server upon publication of the results based on this data. All experimental data that can be reused by others, including all data used in publications will be stored on TU Delft data repository.

3.   After the project has been completed, how will the data be stored for the long-term and made available for the use by third parties? To whom will the data be accessible?

Along with all publications from this project, we will provide open-source code that can be used to re-generate the identical simulation data reported in publications, thereby strongly reducing the need to store large amounts of simulation data over a long time period. The experimental data gathered in the project will be securely stored at 3TU.Datacentrum (http://datacentrum.3tu.nl).

4.   Which facilities (ICT, (secure) archive, refrigerators or legal expertise) do you expect will be needed for the storage of data during the research and after the research? Are these available?*

The required ICT infrastructure is present at TU Delft for storing during the research. Later transfer to the 3TU.Datacentrum is foreseen. TU Delft ftp servers can function to maintain long-term access to source code (as is the case for 15+ years for our scientific Image Processing Library DIPlib/DIPimage via www.diplib.org). Together with Erik Meijering (Erasmus MC) I am involved in the Data Management node of NL-bioimaging which has been funded for bridge financing of the NWO roadmap for large infrastructure in the Netherlands.

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Cost estimates

3a. Budget (in k€)

	Description			Year 1	Year 2	Year 3	Year 4	Year 5	Total
Staff		FTE	Months						
WP	Applicant	0.4	60	50	52	54	57	59	272
WP	PhD1	1.0	48	44	52	55	59		210
WP	PhD2	1.0	48		45	53	56	59	213
WP	PhD3	1.0	48	44	52	55	59		210
WP	PostDoc	1.0	24	64	68				132
OBP	Technician	0.5	24	37	37				74
Total Staff		4.9	252	239	306	217	231	118	1111
Equipment	Dedicated multi-GPU server			20					20
Equipment	Cryo stage			80					80
Equipment	4pi microscope components, table & laser			110					110
Equipment	2 sCMOS cameras			25					25
Equipment	Double aberration correction			65					65
Materials	Optical & vacuum components			30	5	5	5	5	50
Materials	Glass/plastic/chemicals			2	2	2	2	2	10
Materials	Electronics/automation			5	5	5	5		20
Travel	Applicant			2	2	2	2	2	10
Travel	PhDs & PostDoc			6	8	6	6	2	28
Other	Publication costs				3	4	4	4	15
Total Materials				345	25	24	24	15	433
Grand total				584	331	241	255	133	1544

We can make use of an existing Ti:sapphire laser in the lab for STED (price ~120kE).

3b. Contributions 'in kind'

Co-financer/party	Description	Estimated value in Euros
Delmic BV	Hardware and software development for cryo-stage (400 h)	44.400

3c. Contributions 'in cash'

None.

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3d. Totals

Grand total	1.544.400
Budget requested from NWO	1.500.000

3e. Have you applied for any additional grants for this project either from NWO or from any other institution, and/or has the same idea been submitted elsewhere?

No.

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Curriculum vitae

4a. Personal details
Title(s), initial(s), first name, surname: Prof. Dr. B. (Bernd) Rieger

4b. Master's ('Doctoraal')
University/College of Higher Education: Technische Universität München
Date (dd/mm/yy):   16/07/1999
Main subject: Physics

4c. Doctorate
University/College of Higher Education: Technische Universiteit Delft
Starting date (dd/mm/yy):   01/10/1999
Date of PhD award (dd/mm/yy):   09/02/2004
Supervisor ('Promotor'):   Prof.dr.ir. L.J. van Vliet
Thesis title: Structure from Motion in nD Image Analysis

4d. Work experience since completing your PhD

Position	Period	FTE	Position	Institution
Professor*****	01/2017-	1.0	Permanent	TU Delft
Associate Professor	06/2014-12/2016	0.8	Permanent	TU Delft
Assistant Professor	05/2006-05/2014	0.8	Tenure track	TU Delft
Senior Researcher	05/2006-12/2010	0.2	Permanent	FEI Company, Eindhoven
Senior Researcher	05/2005-04/2006	1.0	Permanent	FEI Company, Eindhoven
Post-doc	01/2004-04/2005	1.0	Fixed term	MPI for Biophysical Chemistry, Göttingen (NWO TALENT scholarship)

* I am appointed 'Antoni van Leeuwenhoek professor' which is an honorary early-promotion position for young outstanding researchers at TU Delft.

Months spent since completing your PhD

Experience	Number of months
Research activities	90.52
Education	34.74
Care or sick leave	14.40 (taking care of my children 1d/week)
Management tasks	6.54
Other (please specify)	22.80 (working for FEI Company)

Calculation:
Research: 1.0*16*100% + 0.8*126*65% + 1.0*15*60% = 90.52
Education: 1.0*16*0%   + 0.8*126*30% + 1.0*15*30% = 34.74
Management: 1.0*16*0%   + 0.8*126*5%   + 1.0*15*10% = 6.54
Child care: 0.2*72 =14.40
Other: 1.0*12 + 0.2*54 = 22.80
Total: 169 months ~ 14y

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4e. Academic staff supervised

	Give names or numbers	**Please indicate/specify your role (for PhDs, mark one role)**		
PhDs		Promotor (formal supervisor)	Co-promotor (formal co-supervisor)	Role as (co-) supervisor
Ongoing	*Sabri Bolkar*	X		
	Christiaan Hulleman	X		
	Yan Guo	X		
	Hamid Heydarian	X		
	Rasmus Thorsen	X		
Successfully completed	*Nadya Chakrova*		X	
	Carlas Smith		X	
	Robert Nieuwenhuizen		X (cum laude)	
	Lennart Voortman		X	
	Sanneke Brinkers		X	
	Heidi Dietrich		X	
	Milos Vulovic		X	
Subtotal PhDs	*12*	*5*	*7*	*0*
Postdocs				
Ongoing	*Taylor Hinsdale*	*Supervisor*		
	Robert Moerland	*Supervisor*		
Completed	*Richard Aveyard*	*Supervisor*		
	Vincent van Ravesteijn	*Supervisor*		
	Melanie Kessels	*Supervisor*		
Subtotal postdocs	*5*			
Support staff members				
Subtotal support staff	*5*	*Support staff supervisor*		

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4f. Brief summary of your research over the last five years

At TU Delft, I lead a research team of 6 researchers working on *Computational Microscopy* in light and electron microscopy. It comprises the combination of imaging physics and image processing to surpass fundamental limitations imposed by physics on image formation. My main application area is in life sciences at the molecular level, with the most notable research activity at the moment in localization or super-resolution microscopy, an area also known as optical nanoscopy.

My team aims to develop techniques that offer the highest spatial (and temporal) resolution in microscopic imaging. With this aim, we place ourselves in between purely curiosity and application-driven research. In the framework of my ERC Consolidator Grant project we are currently developing experimental and theoretical means to enable localization microscopy at cryogenic temperature.

In 2013, I introduced a novel resolution concept and measure for nanoscopy (Nieuwenhuizen2013). This measure is now the baseline in the field and used e.g. in the "localization challenge" (Chenouard *et al*., *Nature Methods* 2014). I provided code to accelerate dissemination and gave many invited talks on the subject.

In 2012, I introduced the concept of "data fusion" to the field of localization microscopy. This technique allows sub-nanometer precision measurements with light on reconstructions from many chemically identical entities. This idea has been used to resolve a long-standing debate about the chemical composition of the Nuclear Pore Complex that eluded cryo-electron microscopy (Szymborska *et al., Science* 2013). My Vici project will lift this concept from its fledgling stages to a mature field.

4g. International activities

International Conferences and Workshops:
- Organizer of the 9th *Single Molecule Localization Symposium*, Aug. 2019 in Delft, www.smlms.org. Each year ~150 participants in combination with a small company exhibition.
- Initiator and Organizer of the conference series *Quantitative Bioimaging* since 2013 www.quantitativebioimaging.com. Each year we attract ~150-200 attendees, ~15 international recognized invited speakers. 2013 & 2014 in Albuquerque, USA; 2015 at Institute Pasteur, Paris, France and 2016 at TU Delft. I attracted W.E. Moerner to Delft as keynote speaker, Noble prize laureate 2014 in chemistry. (2017 in Texas, USA and 2018 in Göttingen, Germany)
- Associate Editor for International Symposiums on Biomedical Imaging ISBI 2016, 2017 & 2018, abstract and short paper selection
- Organizer of the "localisation challenge" for single-molecule localization microscopy benchmarking, EPFL 2015
- Keynote lecture at European Bioimaging Conference Eubias, Paris, France, January 5-6, 2015
- Organizer and Lecturer on *Localization Microscopy* at the 22nd Int. Conf. on Pattern Recognition ICPR 2014, Stockholm, Sweden

International Research Activities:
- Visiting scientist for six (2007) and four weeks (2010) at the Department of Physics and Astronomy, University of New Mexico at Albuquerque, USA

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- Member of COST action *CA15124 - A new Network of European BioImage Analysts to advance life science imaging (NEUBIAS).* I have been part of and supported the application process from the beginning; I'm not in a chairing positing right now.

Major International Collaborations:
- Prof.dr. Ralf Jungmann on *Data fusion for 2D and 3D DNA-origami data*, MPI for Biochemistry, Martinsried, Germany. Data exchange and visits.
- Prof.dr. Keith Lidke on *Image analysis in super-resolution microscopy*, Department of Physics and Astronomy, University of New Mexico at Albuquerque, USA. We exchanged in total 3 graduated and 3 undergraduate students for each ~3 months stay over the years. I visited several times and prof. Lidke stayed 2 months in Delft in spring 2017 for a short sabbatical.
- Prof.dr. Markus Sauer on *Algorithms for counting in super-resolution microscopy*, Biozentrum, University of Würzburg, Germany. Several short visits (1-2 days) over the last years.
- Prof.dr. David Grünwald on *Image analysis for very weak signals from single molecules emitters*, University of Massachusetts Medical School Worcester, USA. We exchanged 3 students each for several months over the last year and prof. Grünwald visited Delft a couple of times in the last years. We had one shared PhD student. We currently jointly lead NIH-funded research on 4D chromatin structure.

Invited Talks (selection of 34 thus far):
- 2nd International Conference On Nanoscopy, ICON, Bielefeld, Germany, February 27 – March 2, 2018
- 19th Linzer Winterschool on Biophysics, Linz, Austria, February 5-7, 2017
- SelectBio: Bioimaging From Cells To Molecules, Cambridge, UK, June 14-15, 2016
- European Bioimaging Conference Eubias, Paris, France, January 5-6, 2015 (*keynote lecture*)
- Royal Society Workshop on Super-resolution microscopy, Leeds, UK, July 3-4, 2014
- 3rd Single Molecule Localization Microscopy Symposium, Frankfurt, Germany, August 28-30, 2013

4h. Other academic activities

Organisational Responsibilities:
- Head of section Quantitative Imaging at TU Delft - since 2018
- Board member and treasurer, Dutch Microscopy Society (NVVM) - since 2010
- Board member and treasurer, Foundation for the promotion of Electron Microscopy in the Netherlands (SEN) - since 2010
- Member of the Career Committee, Faculty of Applied Sciences, TU Delft – since 2018
- Member of the TU Delft Diversity Team (established 2018)
- Member of the Curriculum Committee Applied Physics, TU Delft – since 2014
- Member of the Examination Committee Applied Physics, TU Delft – 2012-2014
- Chairman of the Departmental Safety Committee, TU Delft – 2006-2009
- Organizer of the focus session on Single Molecule Microscopy at the Physics@FOM national conference in Veldhoven, The Netherlands - January 2014

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- Serving on external Ph.D. committees in Netherlands; opponent in Uppsala, Sweden

Reviewing:
- Regular reviewer for many journals (identified as one of the best reviewers for Nature Publishing Group 2015).
- External expert reviewer for trans/national funding agencies: STW, STW HTSM jury, NWO Veni/Vidi; Wageningen University (VLAG Graduate School); Wellcome Trust (UK) for the Collaborative Award and Technology Development Grant; Swiss National Science Foundation (SNSF) for the SNSF Professorships (comparable to Vici); Austrian Science Fund (FWF) for the Open Programmes and START Programme (comparable to NWO-Vidi); Carl-Zeiss Stiftung Jena (comparable to NWO-Veni); Agence Nationale de la Recherche (AAP ANR, France); Fonds Wetenschappelijk Onderzoek – Vlaanderen (FWO, Belgium); Deutsche Forschungsgesellschaft (DFG); Human Frontier Science Programme (HFPS) for research grants

Outreach (selected):
- Lunch lecture on the Nobel Prize in Chemistry 2014 for physics students, Vereniging voor Technische Physica; *350 years of light microscopy in Delft*, Family day of the Faculty of Applied Sciences, TU Delft, 2016;
- Teaching for high-school children (4 days), International School of The Hague, 2012 & 2013;
- Developed an animation on super-resolution microscopy (2017) http://www.youtube.com/watch?v=vh3_qOy2uls&t=5s

Valorisation:
- FEI Company (Eindhoven) implemented my defocus estimation algorithms [Vulović et al. Ultramicroscopy 2012] into their release Electron Microscope analysis software, *AutoCTF;*
- Mapper Lithography (Delft) uses my fast GPU implementation [Smith2010] for spot estimation for auto-alignments of wafers
- Consultant for FEI Company and ALSI, Beuningen (now ASM Pacific Technologies)

Undergraduate Teaching and course material development (selected):
- *System and Signals*, 6 ECTS, mandatory course ~300 Applied Physics & Nanobiology students – since 2006; Principal Instructor since 2010
- *Advanced Digital Image Processing,* 6 ECTS, elective MSc, ~50 students from interdisciplinary engineering backgrounds, since 2017
- *Applied Physics Research labs*, second year Applied Physics: I created the Super-Resolution Microscopy lab programme from scratch – since 2015
- *Computational Science*, 3 ECTS, mandatory course ~250 Applied Physics & Nanobiology students, including a major renewal of the course material – 2007-2015; Principal Instructor 2009-2015
- *Computational Science*, minor Applied Physics, 3 ECTS, mandatory course ~35 students, Principal Instructor incl. course material development – 2009-2015
- *High Resolution Microscopy*, 4 ECTS, mandatory course ~20 Nanobiology students, incl. course material development – since 2015

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- *Introduction to Imaging Systems*, 3 ECTS, elective course ~80 Applied Physics students

Innovation in Education:
- Acquired a TU Delft 'Studiesucces' grant of 3000€ for developing formative digital homework via eLearning for the *Systems and Signals* course module – 2014/2015
- Acquired grassroots funds worth 1000€ to implement the eClicker system towards active student participation during lectures in the *Systems and Signals* course module - 2012

Supervision of Bachelor/Master's student projects:
- Supervised 11 MSc and 19 BSc student thesis projects. R.P.J. Nieuwenhuizen received the biannual prize for best Applied Physics MSc. thesis 2011 and C.S. Smith for the best BSc. thesis 2008.

Graduate Teaching:
- *Biophysics: measuring and modeling biology*, one-week course, French Biophysical Society and the French Microscopy Society, Chamonix, France, 2015
- *Advanced Microscopy*, Casimir Graduate School, Delft and Leiden - since 2013
- *Advanced Microscopy,* two-week EMBO course, Buenos Aires, Argentina, 2006

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4i. Scholarships, grants and prizes

Scholarship/Grant/Prize as formal applicant	Total amount	Amount allocated to my research	Year of award
eScience Center, Pathfinder project	50 k€	50 k€	2016
ERC Consolidator Grant; Optical nanoscopy at 1 nm resolution: far-field fluorescence control at cryogenic temperatures	2000 k€	2000 k€	2015
STW-HTSM, Electron Tomography for next generation Integrated Circuits	760 k€	400 k€	2013
NWO middelgroot, Super-resolution in optical sections	174 k€	174 k€	2008
NWO talent fellowship, Quantitative Imaging for Molecular Biology	30 k€	30 k€	2004
Scholarship/Grant/Prize Formal co-applicant			
NL-Bioimaging, Bridge Financing, Infrastructure Roadmap NWO	960 k€	140 k€	2018
National Institute of Health, NIH U01, 4DN extension grant	660 k$	180 k$	2017
National Institute of Health, NIH U01, 4DN, Visualizing local and global chromatin architecture, and gene expression in individual cells by structural single-molecule imaging	1000 k$	350 k$	2015
STW-Perspective; Super-resolution microscopy	5600 k€	1200 k€	2012
BSIK/CONDOR; Image Processing for Scanning Electron Microscopy	2500 k€	290 k€	2010
FOM-FEI-IPP; Ultra-resolution in 3D-cryo TEM	2500 k€	590 k€	2009

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Output

5a. Output indicators

My research field is highly interdisciplinary. I am active in the development of theory, algorithms and applications related to *computational microscopy*, with my main focus on *optical nanoscopy in molecular cell biology*. In all of these fields, the main output indicators are <u>peer-reviewed publications</u> in journals and conferences and <u>citations</u> thereof.

My publications span across many domains. Depending on the type of research work, results can be published in engineering journals, physics journals, computer science journals and a range of applied journals (mostly molecular biology).
In summary, my work thus far has been cited 2800 times (*h*-index 25, *i10*-index 47) according to Google Scholar. Specifically in the domain of image processing, pre-reviewed conference proceedings are also valued and highly competitive. I therefore do not give impact factors.

Finally, in my field, software methods and development are also important. Making them <u>freely available</u> to users in a suitable fashion is a prerequisite for impact. I have been providing output in the format of DIP*lib*/DIP*image* since 2000. DIP*image* is a MATLAB toolbox for scientific image processing and analysis based on the C-library DIP*lib* (www.diplib.org, 1000+ downloads/year); I know of at least 20 academic groups actively using this software. Providing free software and maintaining a large package is a costly and time consuming enterprise not "rewarded" in citations typically, but very important for the community. Therefore I provide free implementations of all of my algorithms from publications in MATLAB, but also GPU code or ImageJ plugins where appropriated.

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5b. Top publications

Number of citations (Google Scholar) denoted between brackets. **[S]** = significant to the current proposal. Full publication list: http://homepage.tudelft.nl/z63s8/publications

1) **Template-free 2D particle fusion in localization microscopy** [**S**]
   H. Heydarian, F. Schueder, M.T. Strauss, B. v. Werkhoven, M. Fazel, K.A. Lidke, R. Jungmann, S. Stallinga, B. Rieger
   Nature Methods, in print, 2018.
   Developed an all-to-all registration approach that enable high-resolution, template-free single particle reconstruction from localization microscopy data. Initiated and directed the research, lead the collaboration between Delft, Munich and USA.

2) **Measuring image resolution in optical nanoscopy** [**S**]
   R.P.J. Nieuwenhuizen, K.A. Lidke, M. Bates, D. Leyton Puig, D. Grünwald, S. Stallinga, B. Rieger
   Nature Methods, 10(6): 557-562, 2013. [240]
   Found a way to assess resolution in optical nanoscopy; provided a software solution that is currently widely used. Initiated and directed the research, lead the collaboration between Delft, Amsterdam, USA and Göttingen.

3) **Image formation modeling in Cryo Electron Microscopy**
   M. Vulović, R.B.G. Ravelli, L.J. van Vliet, A.J. Koster, I. Lazić, U. Lücken, H. Rullgård, O. Öktem, B. Rieger
   Journal of Structural Biology, 183(1):19-32, 2013. [41]
   Developed a simulation framework and validated that with cryo-EM experiments; provided software package InSilicoTEM. Initiated and directed the research, lead the collaboration between Delft, Leiden, FEI Company and Stockholm.

4) **Fast, single-molecule localization that achieves theoretically minimum uncertainty** [**S**]
   C.S. Smith, N. Joseph, B. Rieger*, K.A. Lidke*
   Nature Methods, 7(5):373-375, 2010. [343]
   First to show that theoretical optimal fitting can be done in real time; provided a software solution that is still much used. Initiated the research together with K. Lidke, sent my undergrad student C. Smith to work in his lab for 3 months.

5) **Superresolution by localization of quantum dots using blinking statistics**
   K.A. Lidke*, B. Rieger*, T.M. Jovin, R. Heintzmann
   Optics Express, 13(18):7052-7062, 2005. [326]
   Found the algorithmic breakthrough for separating quantum dots positions from their intermittent emission. Keyed the term pointillism for the image formation.

5c. Output (selection)
6) [**S**] C. Hulleman, M. Huisman, R. Moerland, D. Grünwald, S. Stallinga, B.Rieger, *Fluorescence polarization control for on-off switching of single molecules at cryogenic temperatures*, Small Methods, 1700323, 2018.
7) [**S**] C.H. Hulleman, W. Li, I. Gregor, B. Rieger, J. Enderlein. *Photon yield enhancement of red fluorophores at cryogenic temperatures*. ChemPhysChem, 19:1774-1780, 2018.
8) R.Ø. Thorsen, C.N. Hulleman, M. Hammer, D. Grünwald, S. Stallinga, B. Rieger, *Photon count estimation in single-molecule localization microscopy*, Nature

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Methods, under revision, 2018.

9) R. Aveyard, Z. Zhong, K.J. Batenburg, <u>B. Rieger</u>, *Optimizing experimental parameters for the projection requirement in HAADF-STEM tomography*, Ultramicroscopy, 177:84-90, 2017. [1]

10) N. Chakrova, <u>B. Rieger</u>, S. Stallinga, *Deconvolution methods for structured illumination microscopy*, Journal of the Optical Society of America A, 33, 2016. [14]

11) C.S. Smith, S. Preibisch, A. Joseph, S. Abrahamsson, <u>B. Rieger</u>, E. Myers, R.H. Singer, and D. Grünwald, *Nuclear accessibility of β-actin mRNA measured by 3D single molecule real time (3D-SMRT) microscopy*, J. Cell Biology, 209, 2015. [26]

12) [**S**] J. Broeken, H. Johnsson, D.S. Lidke, L. Sheng, R.P.J. Nieuwenhuizen, S. Stallinga, K.A. Lidke, <u>B. Rieger</u>, *Resolution improvement by 3D particle averaging in localization microscopy*, Meth. Appl. Fluo., 3:014003, 2015. [25]

13) [**S**] <u>B. Rieger</u>, S. Stallinga, *The lateral and axial localization uncertainty in super-resolution light microscopy*, ChemPhysChem, 15, 2014. [47]

14) [**S**] L.M. Voortman, M. Vulović, M. Maletta, A. Voigt, E.M. Franken, A. Simonetti, P.J. Peters, L.J. van Vliet, <u>B. Rieger</u>, *Quantifying resolution limiting factors in subtomogram averaged cryo-electron tomography using simulations*, J. Struct. Biology, 187, 2014. [13]

15) [**S**] S. Stallinga, <u>B. Rieger</u>, *Position and orientation estimation of fixed dipole emitters using an effective Hermite PSF model*, Opt. Exp., 20, 2012. [29]

16) [**S**] A. Löschberger, S. van de Linde, M.-C. Dabauvalle, <u>B. Rieger</u>, M. Heilemann, G. Krohne, M. Sauer, *Super-resolution imaging reveals eightfold symmetry of gp210 proteins around the nuclear pore complex and resolves the central channel with nanometer resolution*, J. Cell Science, 125, 2012. [174]

17) L.M. Voortman, S. Stallinga, R.H.M. Schoenmakers, L.J. van Vliet, <u>B. Rieger</u>, *A fast algorithm for computing and correcting the CTF for tilted, thick specimens in TEM*, Ultramicroscopy, 111:1029-1036, 2011. [20]

18) [**S**] S. Stallinga, <u>B. Rieger</u>, *Accuracy of the Gaussian point spread function model in 2D localization microscopy*, Optics Express, 18, 2010. [131]

19) S. Brinkers, H.R.C. Dietrich, F.H. de Groote, I.T. Young, <u>B. Rieger</u>, *The Persistence Length of Double Stranded DNA Determined Using Dark Field Tethered Particle Motion*, J. Chemical Physics, 130, 2009. [86]

20) D.S. Lidke, K.A. Lidke, <u>B. Rieger</u>, T.M. Jovin, D.J. Arndt-Jovin, *Reaching out for signals: Filopodia act as sensory organs via retrograde transport of activated EGF receptors*, J. Cell Biology, 170, 2005. [232]

21) K.A. Lidke*, <u>B. Rieger</u>*, D.S. Lidke, T.M. Jovin, *The role of photon statistics in fluorescence anisotropy imaging*, IEEE Trans. Image Processing, 14, 2005. [64]

22) <u>B. Rieger</u>, F.J. Timmermans, L.J. van Vliet, P.W. Verbeek, *On curvature estimation of surfaces in 3D grey-value images and the computation of shape descriptors*, IEEE Trans. Pattern Analysis and Machine Intelligence, 26, 2004. [46]

23) <u>B. Rieger</u>, L.J. van Vliet, *Curvature of n-dimensional Space Curves in Grey-value Images*, IEEE Trans. Image Processing, 11, 2002. [30]

Total numbers of output items:		
- **Refereed articles:**	**70**	
- **Non-refereed articles:**	**5**	(e.g. Nederlandse Tijdschrift voor Natuurkunde)
- **Book chapters:**	**2**	
- **Patents:**	**4**	(including an ISO standard ISO/TS 24597:2011)

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5d. Median impact factors for your field: N/A

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Statements by the applicant

Use of extension clause: no

Ethical aspects

	Not applicable	Not yet applied for	Applied for	Received
Approval from a recognised (medical) ethics review committee	X			
Approval from an animal experiments committee	X			
Permission for research with the population screening Act	X			

If applicable, proof of approval will need to be sent to NWO before the start of your Vici project.

Declarations
By submitting this form I endorse the code of conduct for laboratory animals and the code of conduct for biosecurity/possibility for dual use of the expected results and will act accordingly if applicable.

☑   I have completed this form truthfully

☑   By submitting this document I declare that I satisfy the nationally and internationally accepted standards for scientific conduct as stated in the *Netherlands Code of Conduct for Scientific Practice 2014* (Association of Universities in the Netherlands)

☐   If applicable: I have submitted a list of non-referees with my pre-proposal.[1]

☑   If applicable: I have included one or more authorised letters from the host institution (or a third party) guaranteeing to meet part of the costs of this research project.

Name: Bernd Rieger

Place: Delft

Date: 27 Augustus 2018

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[1] When you submit your full proposal you cannot submit (new) non-referees.

Vernieuwingsimpuls
Innovational Research Incentives Scheme
Grant application form (full proposal) 2018   **Vici scheme**

Ultra-resolution with visible light   *Bernd Rieger*

Please submit this <u>signed</u> application form to NWO in <u>PDF format. For applications to all domains except ZonMw</u> use the ISAAC system. For applications to ZonMw, use the ProjectNet system. Please do not use any security locks or bookmarks in the PDF file. For any technical questions regarding submission, please contact the ISAAC helpdesk (isaac.helpdesk@nwo.nl) or the ProjectNet helpdesk (projectnet@zonmw.nl), respectively.