Single-molecule localization algorithms in super-resolution microscopy

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Abstract

Localization microscopy is a class of super-resolution fluorescence microscopy techniques. Localization microscopy methods are characterized by stochastic temporal isolation of fluorophore emission, i.e., making the fluorophores blink so rapidly that no two are likely to be photoactive at the same time close to each other. Well-known localization microscopy methods include *d*STORM, STORM, PALM, FPALM, or GSDIM. The biological community has taken great interest in localization microscopy, since it can enhance the resolution of common fluorescence microscopy by an order of magnitude at little experimental cost. However, localization microscopy has considerable computational cost since millions of individual stochastic emissions must be located with nanometer precision. The computational cost of this evaluation, and the organizational cost of implementing the complex algorithms, has impeded adoption of super-resolution microscopy for a long time.

In this work, I describe my algorithmic framework for evaluating localization microscopy data. I demonstrate how my novel open-source software achieves real-time data evaluation, i.e., can evaluate data faster than the common experimental setups can capture them. I show how this speed is attained on standard consumer-grade CPUs, removing the need for computing on expensive clusters or deploying graphics processing units. The evaluation is performed with the widely accepted Gaussian point spread function (PSF) model and a Poissonian maximum-likelihood noise model.

I extend the computational model to show how robust, optimal two-color evaluation is realized, allowing correlative microscopy between multiple proteins or structures. By employing cubic B-splines, I show how the evaluation of three-dimensional samples can be made simple and robust, taking an important step towards precise imaging of micrometer-thick samples. I uncover the behavior and limits of localization algorithms in the face of increasing emission densities.

Finally, I show up algorithms to extend localization microscopy to common biological problems. I investigate cellular movement and motility by considering the in vitro movement of myosin-actin filaments. I show how SNAP-tag fusion proteins enable imaging with bright and stable organic fluorophores in live cells. By analyzing the internal structure of protein clusters, I show how localization microscopy can provide new quantitative approaches beyond pure imaging.

Zusammenfassung

Lokalisationsmikroskopie ist eine Methodenklasse der superauflösenden Fluoreszenzmikroskopie, deren Methoden sich durch stochastische zeitliche Isolation der Fluoreszenzemission auszeichnen. Das Blinkverhalten von Fluorophoren wird so verändert, dass gleichzeitige Aktivierung von einander nahen Fluorophoren unwahrscheinlich ist. Bekannte lokalisationsmikroskopische Methoden umfassen *d*STORM, STORM, PALM, FPALM, oder GSDIM. Lokalisationsmikroskopie ist von hohem biologischem Interesse, weil sie die Auflösung des Fluoreszenzmikroskops bei minimalem technischem Aufwand um eine Größenordnung verbessert. Der verbundene Rechenaufwand ist allerdings erheblich, da Millionen von Fluoreszenzemissionen einzeln mit Nanometergenauigkeit lokalisiert werden müssen. Der Rechen- und Implementationsaufwand dieser Auswertung hat die Verbreitung der superauflösenden Mikroskopie lange verzögert.

Diese Arbeit beschreibt meine algorithmische Grundstruktur für die Auswertung lokalisationsmikroskopischer Daten. Die Echtzeitfähigkeit, d.h. eine Auswertegeschwindigkeit oberhalb der Datenaufnahmegeschwindigkeit an normalen Messaufbauten, meines neuartigen und quelloffenen Programms wird demonstriert. Die Geschwindigkeit wird auf verbrauchermarktgängigen Prozessoren erreicht und dadurch spezialisierte Rechenzentren oder der Einsatz von Grafikkarten vermieden. Die Berechnung wird mit dem allgemein anerkannten Gaussschen Punktantwortmodell und einem Rauschmodell auf Basis der größten Poissonschen Wahrscheinlichkeit durchgeführt.

Die algorithmische Grundstruktur wird erweitert, um robuste und optimale Zweifarbenauswertung zu realisieren und damit korrelative Mikroskopie zwischen verschiedenen Proteinen und Strukturen zu ermöglichen. Durch den Einsatz von kubischen Basissplines wird die Auswertung von dreidimensionalen Proben vereinfacht und stabilisiert, um präzisem Abbilden von mikrometerdicken Proben näher zu kommen. Das Grenzverhalten von Lokalisationsalgorithmen bei hohen Emissionsdichten wird untersucht.

Abschließend werden Algorithmen für die Anwendung der Lokalisationsmikroskopie auf verbreitete Probleme der Biologie aufgezeigt. Zelluläre Bewegung und Motilität werden anhand der *in vitro* Bewegung von Myosin-Aktin-Filamenten studiert. Lebendzellbildgebung mit hellen und stabilen organischen Fluorophoren wird mittels SNAP-tag-Fusionsproteinen realisiert. Die Analyse des Aufbaus von Proteinklumpen zeigt, wie Lokalisationsmikroskopie neue quantitative Ansätze jenseits reiner Bildgebung bietet.

Contents

Abstract v

Zusammenfassung vii

1 Introduction 1
- 1.1 Motivation and Overview . . . 1
- 1.2 Classical microscopy . . . 2
  - 1.2.1 Illumination techniques . . . 3
  - 1.2.2 Providing contrast . . . 4
  - 1.2.3 Fluorescence microscopy . . . 4
  - 1.2.4 Photochemistry of fluorophores . . . 5
  - 1.2.5 Electron microscopy . . . 7
  - 1.2.6 Short-distance energy transfer . . . 8
- 1.3 Super-resolution microscopy . . . 8
  - 1.3.1 Single-molecule localization methods . . . 10
- 1.4 Cellular biology . . . 12
- 1.5 Single-molecule localization algorithms . . . 12
  - 1.5.1 The Gaussian point spread function model . . . 13
  - 1.5.2 Least squares fitting and Levenberg-Marquardt . . . 14
  - 1.5.3 Maximum-likelihood fitting . . . 15
- 1.6 Software development . . . 16
  - 1.6.1 Prior and concurrent art . . . 16
  - 1.6.2 Free, open source and open access software . . . 17
  - 1.6.3 Agile development . . . 18

2 Articles 19

Real-time computation of subdiffraction-resolution fluorescence images . . . 20

Direct stochastic optical reconstruction microscopy with standard fluorescent probes . . . 32

Follow-up to paper by S. Wolter, M. Schuttpelz, M. Tscherepanow, S. van de Linde, M. Heilemann and M. Sauer, entitled Real-Time Computation of Subdiffraction-Resolution Fluorescence Images . . . 52

rapidSTORM: accurate, fast open-source software for localization microscopy 54

Cubic B-spline calibration for 3D-Superresolution measurements . . . 77

Data Analysis for Single-Molecule Localization Microscopy . . . 91

The effect of photoswitching kinetics and labeling densities on super-resolution fluorescence imaging . . . 112

Measuring localization performance of super-resolution algorithms on very active samples . . . 120
Subdiffraction-Resolution Fluorescence Microscopy of Myosin–Actin Motility 135
Live-cell dSTORM with SNAP-tag fusion proteins . . . 146
Super-resolution imaging reveals the internal architecture of nano-sized syntaxin clusters . . . 157

3 Discussion **171**
3.1 Software creation . . . 171
3.2 Three-dimensional evaluation . . . 172
3.3 Multi-color and multi-plane evaluation . . . 173
3.4 High-density problem . . . 173
3.5 Biological applications . . . 174

4 Conclusion and Outlook **177**

A Ehrenwörtliche Erklärung **179**

B Acknowledgements **181**

C Acronyms **183**

D rapidSTORM downloads **185**

Chapter 1

Introduction

> We often frame our understanding of what the [instrument] will do in terms of what we expect to find, and actually it would be terribly anticlimactic if in fact we find what we expect to find. [...] The most important discoveries will provide answers to questions that we do not yet know how to ask and will concern objects we have not yet imagined.
>
> — *John Norris Bahcall, NY Times 08/19/2005*

1.1 Motivation and Overview

Fluorescence microscopy is so fundamental to biological research that Ehrenberg [2008] spoke of a "revolution in the biological sciences" when supporting the Nobel prize for a single fluorescent marker, the green fluorescent protein (GFP). While fluorescent markers make single biomolecules visible, classical light microscopy is limited by diffraction to a spatial resolution of roughly 200 nm.

With typical procaryote cell sizes in the 1–5 μm range and eucaryote cells in the 10–100 μm range, this resolution is sufficient to see individual cells and their macrostructure. However, many important cell organelles are a single order of magnitude smaller than the diffraction limit, such as mitochondria with 100–500 nm width [Frey and Mannella, 2000], microtubuli with $\sim$ 50 nm [Heilemann et al., 2008] and the cell membrane with $\sim$ 10 nm. Many macromolecules have similar sizes, e.g. the ribosome with $\sim$ 20 nm and the RNA polymerase at $\sim$ 10 nm. If the resolution of the optical microscope was improved by a single order of magnitude beyond the diffraction limit, we could observe the substructure of these molecules and see individual biomolecules in their cellular context.

While the diffraction limit is fundamental and cannot be broken [Abbe, 1873], several methods for its circumvention have been established and are known as *super-resolution microscopy*. There is a wide variety of super-resolution microscopy methods, ranging from optical, excitation-based methods such as structured illumination (SIM) to highly fluorophore-specific methods such as super-resolution optical fluctuation imaging (SOFI).

One of the most promising super-resolution methods is single-molecule localization microscopy: By combining physics, chemistry, biology and scientific computing, researchers can trade temporal resolution for a ten-fold increase in spatial resolution.

Single-molecule localization microscopy can be realized on standard wide-field fluorescence microscopes and with common-place chemical and biological knowledge, and has the potential to become a staple technique of the biological laboratory.

The prime obstacle to wide use of single-molecule localization microscopy is the computational effort involved in evaluating single-molecule localization data. Millions of individual, isolated, and stochastic fluorescence emissions (spots) must be detected, localized and displayed with computationally demanding algorithms. The generation of a single small super-resolution image, whose raw data can be acquired within minutes, takes several hours with a naïve implementation [Schüttpelz, 2008] on consumer hardware. This costly computation delays the experimental feedback and is a great hindrance in the biological laboratory. Fast, reliable and easy-to-use software is commercially unavailable and exceeds the scope of ad-hoc development both quantitatively and qualitatively.

In this thesis, I document and demonstrate my progress on fast, reliable and easy-to-use algorithms and software programs to evaluate single-molecule localization microscopy data. Chapter 1 gives the theoretical and experimental context of single-molecule localization microscopy for readers that are unfamiliar with the topic. Chapter 2 contains the major scientific publications I have authored and co-authored on the topic. Each article has its own introduction, which is directed at and sufficient for the experienced reader. Chapters 3 and 4 provide discussion, conclusions and outlooks that surpass the scope of an individual article or were unknown at the original articles' creation time.

1.2 Classical microscopy

The optical microscope is designed to enhance the capacity of the human eye to discern structures in the micrometer domain. The quality of a microscopic image is determined by three criteria: freedom from aberrations, resolution and contrast (Fig. 1.1).

An aberration-free image truthfully reports the spatial relations between the imaged objects. An aberrated image appears distorted, and light of different wavelengths from the same light source is often projected onto different points of the image (*chromatic aberration*). While aberrations are an unavoidable side effect of the use of nontrivial optics, they can be minimized with careful optical design and rarely hinder biological microscopy.

Resolution measures the ability of the microscope to differentiate between spatially close entities. A microscope with perfect resolution maps the signal of point-like light sources in the sample to single points in the image plane, and thereby makes distinction of arbitrarily close points possible. However, the resolution of microscopes is fundamentally limited due to the wave nature of light Abbe [1873], and therefore all microscopes display point-like light sources as a diffraction pattern. The spatial intensity distribution of the diffraction pattern is described by the PSF. The PSF is a property of the microscope and takes the form of an Airy disc for a standard wide-field microscope.

The image of a microscope is the convolution of the observed sample's intensity and the PSF. Details in the resulting convoluted image are limited by the size of the PSF. The mathematical field of deconvolution studies algorithms to revert the process and obtain information about the original intensity distribution from a convoluted image

(a) No image errors (b) Aberrations (c) Low resolution (d) Low contrast

Figure 1.1: Illustration of typical image errors in microscopy. The image of Hans Lipperhey, a microscopy pioneer, was artificially reduced in quality to show the types of typical quality-degrading effects that occur in microscopy.

[Starck et al., 2002].

Microscopic resolution is usually quantified by the Rayleigh criterion, which states that the minimum distance between resolvable light sources is the distance from the central diffraction maximum of the PSF to the first diffraction minimum [Born and Wolf, 1975]. Since the limiting factor of an optical microscope is the aperture of the objective, the limit can be calculated explicitly. The limit depends on the optical density of the medium between object and objective n, on the objective opening half-angle α, and on the detection light's wavelength λ (Eq. 1.1).

$$d = \frac{0.61\lambda}{n \sin \alpha} \tag{1.1}$$

Contrast is the microscope's ability to distinguish structures of interest from background. Typical background sources include stray excitation light, uninteresting biological structures or detector readout noise. The primary complication introduced by high background levels is intensity variance: Intensity levels within the background can fluctuate sufficiently to mask the difference between the structure of interest and the background.

1.2.1 Illumination techniques

Most microscopy methods rely on externally illuminating the sample with *excitation light* and observing the response. The choice of the illumination method determines which part of the sample can be detected, and thereby can greatly enhance contrast and resolution.

Widefield illumination provides approximately constant illumination over the whole field of view and the whole depth of the sample. The advantage is that the whole sample can, within the focal limits of the optics, be viewed at once. The disadvantage is that the parts of the sample that are not in the detection focus contribute considerable noise to the image. Widefield illumination is easy to achieve and the standard approach in microscopy.

total internal reflection (TIR) illumination limits the illumination to an evanescent field close to the cover slip. By angling the illumination beam over the

critical angle of the boundary between cover slip and sample, the illumination light is reflected at the boundary. However, an evanescent electromagnetic field penetrates into the sample. The intensity of the evanescent field decreases exponentially in the axial direction. Consequently, the effective range of illumination is limited to up to $\sim$ 200 nm from the cover slip [Ambrose, 1956]. TIR microscopy combined with fluorescence microscopy is abbreviated as TIRF microscopy.

Dirty-TIR illumination provides illumination along a thin, oblique sheet of light by angling the illumination beam close to the critical angle of the boundary between cover slip and sample. Contrary to true TIR illumination, dirty-TIR lets illumination light penetrate into the sample. Dirty-TIR is also known as highly inclined and laminated optical sheet (HILO) [Tokunaga et al., 2008], critical angle fluorescence microscopy (CAFM) [Nakata and Hirokawa, 2003] or low-angle oblique (LAO) [Sako, 2006].

Point illumination limits illumination to a diffraction limited region in the sample. It is used in *confocal microscopy* together with single-point detection to increase both resolution and contrast at the cost of seeing only a minimal, spatially unresolved volume of the sample at any given time [Minsky, 1955].

1.2.2 Providing contrast

Since acceptable levels of aberration and resolution close to the Abbe limit can be achieved fairly easily with optical components, the primary achievements of biological microscopy have been made in the improvement of contrast. The primary methods in microscopy are therefore distinguished by the physical effect that provides contrast.

Bright-field microscopy relies on absorption and is the basic microscopy technique. The sample is uniformly illuminated from behind (standard) or above (*inverted* microscope), and the image appears dark where light is absorbed by the sample. Many biological samples show poor contrast in bright-field microscopy.

Polarized light microscopy relies on polarization change. Excitation light is polarized, and the detection path is polarization-sensitive [Inoué and Oldenbourg, 1998].

Dark-field microscopy relies on light scattering in the sample. Transmitted light is blocked from the detection path and contrast is improved at the expense of signal intensity [Kudo et al., 1990].

Phase-contrast microscopy relies on the phase contrast difference due to different optical densities of sample and medium [Hughes and Swann, 1948].

Fluorescence microscopy relies on the Stokes shift and is described in detail in section 1.2.3.

1.2.3 Fluorescence microscopy

Fluorescence is an optical phenomenon where the absorption of light by a fluorescent sample causes the emission of longer-wavelength light within nanoseconds. Fluorescence has been known since the 19th century [Herschel, 1845, Stokes, 1852] and the

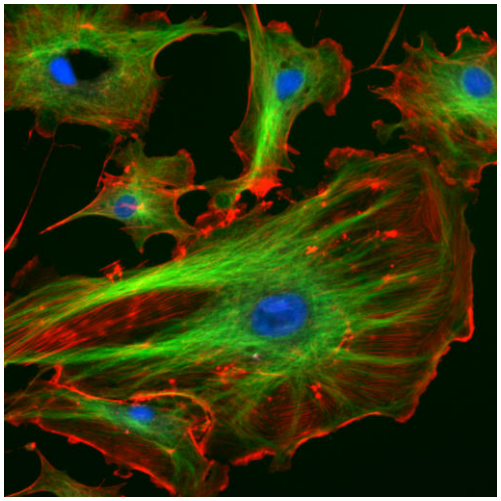

Figure 1.2: Bovine pulmonary artery endothelial cells under the fluorescence microscope. Nuclei are stained blue, microtubuli are stained green and actin filaments are stained red [Rasband, 1997-2008].

fluorescence microscope has been a core tool of cellular biology since the 20th century. The importance of fluorescent proteins has been so central to biological microscopy that the chemistry Nobel prize in 2008 has been awarded to Osamu Shimomura, Martin Chalfie, and Roger Y. Tsien for the discovery of GFP.

Biological fluorescence microscopy is performed by labelling cellular structures with fluorescent dyes, illuminating the sample with short-wavelength light and viewing it through a suitable long-pass filter so that only the light emitted by fluorophores is visible. Since wild-type cells show very little fluorescence, the artificially labelled structures become visible with very high contrast and specificity.

Labelling is usually performed by attaching fluorescent antibodies to the structures of interest (*immunofluorescence*) or by inserting recombinant deoxyribonucleic acid into the observed organism to cause expression of fluorescent proteins that are spliced to the proteins of interest. For economy reasons, the immunofluorescence approach is performed with a pair of antibodies, with a primary antibody that specifically targets the structure of interest and is produced by a host organism such as goat, mouse or rabbit, and a secondary antibody that targets the host organism and is fluorescent. Section 2.2 gives a detailed overview over labelling techniques.

1.2.4 Photochemistry of fluorophores

Fluorophore behavior is characterized through four states in which molecules may reside (Fig. 1.3):

Inactive fluorophores show no fluorescence. Their activation into the bright state can be performed through irradiation (*photoactivation*) or through physical, chemical or biological processes.

Bright fluorophores currently show fluorescence.

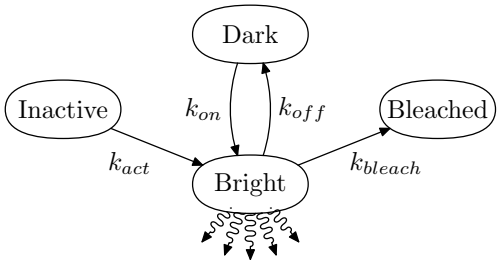

Figure 1.3: Abstract fluorophore model. A fluorophore can be in one of the four states of being inactive, dark, bleached or bright. The transitions from inactive to bright and bright to bleached are irreversible. All allowed transitions have associated rates k. Super-resolution methods mostly use a subset of this graph: photoactivation methods do not use a dark state, but rather the transition from inactive to bleached, while photoswitching methods focus on the dark-bright-dark transitions while not actively using photobleaching.

Dark fluorophores currently show no fluorescence, but can switch into the bright state through chemical reactions. Irradiation may be used to induce the transition, but is often not necessary. Cycling a fluorophore between the bright and the dark state is called *photoswitching*.

Bleached fluorophores have ceased to show fluorescence and cannot be recovered into the bright state. When irradiation causes fluorophores to go into the dark state, the process is called *photobleaching*.

The behavior of synthetic organic dyes of the carbocyanine, oxacine and rhodamine families is well understood, and the transition mechanisms are well known. These molecules show fluorescence through a dislocalized electron [Kuhn, 1948]. The electron is excited from its ground state (S_0) by the excitation light and reaches an excited singlet state. Through a combination of non-radiative relaxation of vibrational energy and light emission, the electron can return to the ground state. Due to the Franck-Condon principle [Franck and Dymond, 1926, Condon, 1926], the excitation process will favor vibrational levels that have a high wave function overlap between the ground and the excited state, and excitation into a both both electronically and vibrationally excited state is the norm. The density of vibrational states facilitates a quick dissipation of the vibrational part into the local environment, while the electronic part is re-emitted as a photon. Because of the loss of vibrational energy, the emitted photon has a longer wavelength than the absorbed photon had. This effect is known as the Stokes shift.

While the singlet cycle is the most likely path, an electron in the excited singlet state can perform *inter-system crossing*, i.e. revert its spin to produce a triplet state. While the triplet state is energetically unfavorable, its decay into the singlet state is forbidden by quantum mechanics and therefore a very slow process [Fujisawa et al., 2002]. While the singlet cycle is usually performed in nanoseconds, the triplet cycle is on the order of microseconds or even milliseconds. This long lifetime in an excited state makes reaction with radicals likely, and the product is nonfluorescent.

When no recovery of the oxidized or reduced state is possible, this reaction is known as *photobleaching* and is usually an undesired effect, because it reduces the

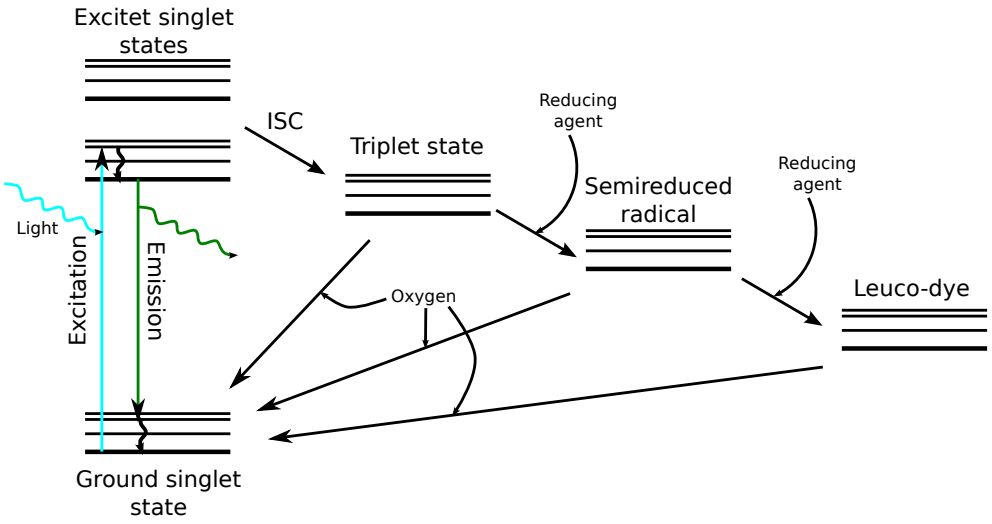

Figure 1.4: Typical Jablonski diagram for a switchable fluorescent dye [van de Linde et al., 2011a]. The majority of the dye population rests in the ground state. Excitation light pushes single molecules into an excited singlet state, and they usually return through a combination of internal, nonradiative relaxation and fluorescence emission. Sometimes, the excited singlet state will instead transmute into a triplet state, i.e. have parallel instead of antiparallel spins on the active electrons, with a considerably longer lifetime than the singlet state. The triplet state is both long-lived and radical, and can react with reducing agents to form semireduced or fully-reduced forms that have lifetimes on the scale of seconds and do not show fluorescence. Oxygen can recover the triplet and the radical states and thereby has considerable influence on the fluorescence.

population of available fluorophores. Kottke et al. [2010] and van de Linde et al. [2011a] have shown that chemical recovery of the oxidized or reduced state is possible under suitable buffer conditions, and the resulting reaction is known as *photoswitching*. The recovery time of the ground state from the oxidized or reduced state is usually on the order of seconds, nine orders of magnitude slower than the fluorescence process.

1.2.5 Electron microscopy

Eq. 1.1 gives a simple, scalable approach to enhance the resolution: reducing the wavelength of the detection particle. By using short-wavelength light such as ultraviolet light, X rays or even γ rays, the resolution can easily be enhanced. Ultimately, even atomic particles can be used since they show wave effects due to the particle-wave duality.

Technical implementation details favor the electron microscope over the X ray, γ or proton microscopes. Electron microscopy has a long and successful history in biology, and super-resolution light microscopy techniques must be measured against electron microscopy. Electron microscopy has the distinct advantage of being a well-established technique with comparatively cheap costs (Tbl. 1.1). It is handicapped by the need for expensive and difficult sample preparation that precludes the use on living cells [Bozzola and Russell, 1999], and thereby also prevents dynamic imaging

Table 1.1: Comparative costs of super-resolution microscopes.

Type	Resolution	Manufacturer	Cost
TM-3000 Electron Microscope	30 nm	Hitachi	60,000 $ [Soulskill, 2009]
TCS 4Pi microscope	100 nm	Leica	N/A
Alpha NSOM	25 nm	WITec	210,000 $ [Harris, 2003]
TCS STED microscope	60 nm	Leica	1,000,000 $ [Perkel, 2011]
Blaze SIM microscope	∼120 nm	Applied Precision	500,000 $ [Dance, 2010]
dSTORM microscope	40 nm	Holm et al.	16,000 $

of intracellular processes.

1.2.6 Short-distance energy transfer

The strong dependency of dipole-dipole energy transfers on the distance between donor and acceptor can be exploited to measure the distance between two spectrally differentiable emitters on a nanometer scale. In biological research, Förster resonance energy transfer (FRET) and photoinduced electron transfer (PET) are commonly used. However, energy transfer methods can only give a small number of distances in the sample, with most practical applications limited to measuring one pair distance per diffraction limited region. Therefore, while being generally quite useful, energy transfer methods can not produce super-resolution images.

1.3 Super-resolution microscopy

> Once we add sound, color, and stick Eddie Murphy in there somewhere, it'll be a smash.
>
> — *ALF, Like an old time movie*

Super-resolution microscopy is the umbrella term for microscopy methods that circumvent the diffraction barrier. Super-resolution methods are classified (Fig. 1.5) into *true* super-resolution methods that work emitter-independently and *functional* super-resolution methods that exploit the characteristic behavior of fluorophores. True super-resolution methods include structured illumination (SIM), additional objectives as in 4Pi microscopy (4PI), near-field scanning optical microscopy (NSOM), and pure computational approaches as in the field of geometrical super-resolution methods, all of which are experimentally or computationally quite complex and outside the implementation scope of a biological research group.

Functional super-resolution methods are in general much simpler due to the special photochemistry of many fluorophores described in 1.2.4. Functional super-resolution methods fall into three broad categories:

Saturation methods such as stimulated emission depletion (STED), saturated structured illumination microscopy (SSIM) or ground-state depletion (GSD) use

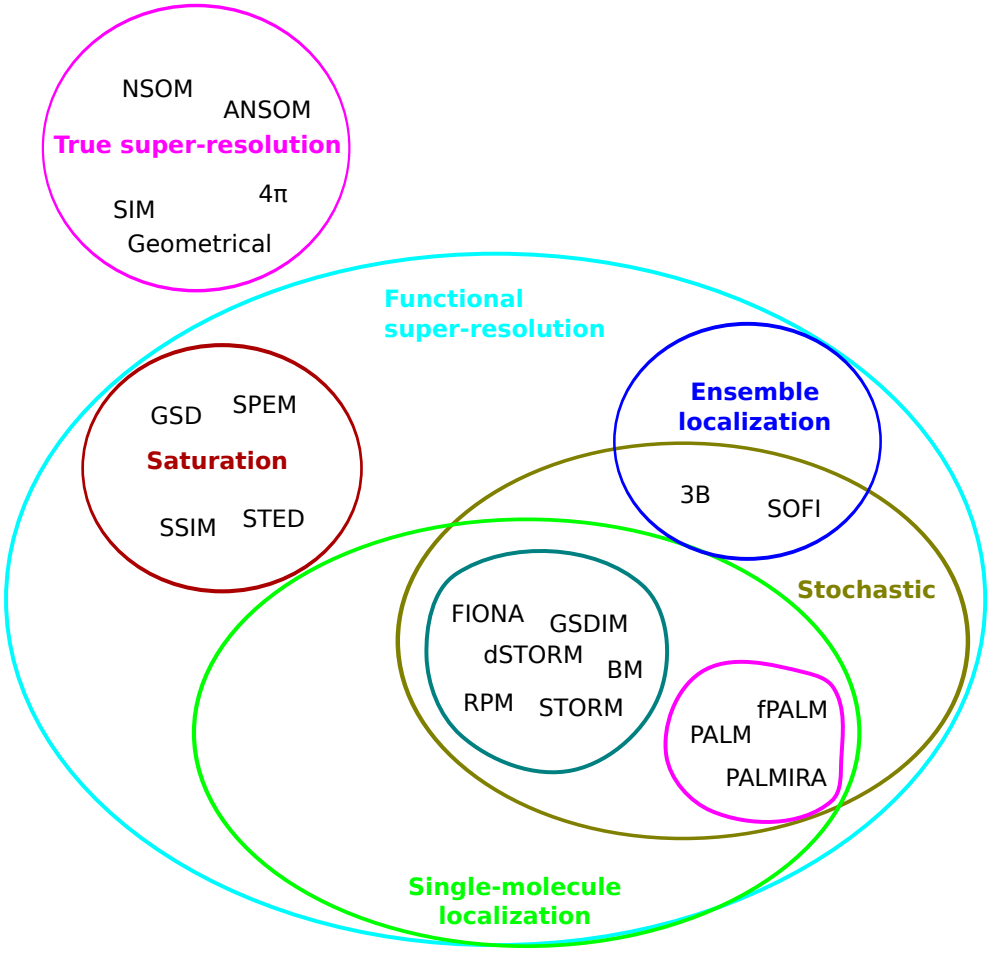

Figure 1.5: Venn diagram of the super-resolution microscopy methods. The teal ellipse groups the methods that apply to synthetic dyes only, while the fuchsia ellipse groups the fluorescent-proteins-only methods.

strong irradiation to saturate the fluorescence process and achieve a qualitative difference between close-by fluorophores. The surviving fluorophore emission is deterministic in time and space. Consequently, saturation-based super-resolution microscopes yield images directly and require no computational post-processing. The saturation methods are also known as deterministic methods or reversible saturable optical flurorescence transitions (RESOLFT).

Single-molecule localization methods such as stochastic optical reconstruction microscopy (STORM), direct stochastic optical reconstruction microscopy (*d*STORM), photoactivated localization microscopy (PALM) or fluorescence photoactivation localization microscopy (FPALM) modify the blinking rates of fluorophores to isolate the individual fluorophore emissions in time. The isolated emissions can be fitted with a model of the PSF with very high precision. The process is stochastic, and computational post-processing is essential, but can be performed in real-time. Single-molecule localization microscopy is also known as AWESOME wide-field excitation super-resolution optical microscopy except SOFI (AWESOME) or spectral precision distance microscopy (SPDM).

Ensemble localization methods such as SOFI and bayesian analysis of blinking and bleaching (3B) observe the natural or slightly modified stochastic blinking of fluorophores and compute likely fluorophore distributions. The isolation of individual fluorophore emissions is not necessary, but computational post-processing is essential and costly.

Single-molecule and ensemble localization methods have a common excitation and acquisition scheme, and raw data from single-molecule localization methods can be evaluated with ensemble localization software, but not vice versa. Therefore, both groups are often summarized as *stochastic* methods in contrast to the *deterministic* saturation methods.

Stochastic methods have a considerable experimental cost advantage over saturation, true optical super-resolution and electron microscopy techniques. Table 1.1 lists the comparative costs of building or buying a relevant microscope. The single-molecule localization microscope beats microscopes with comparable resolution by orders of magnitude. While some of these savings are the natural result of scratch-building a microscope, the *d*STORM microscope is the only one that can be built from very simple components that are already present in many biological groups, and is within the scope of a graduate student. The core complexity of stochastic super-resolution methods is within the evaluation software, and software is easily and cheaply replicated.

1.3.1 Single-molecule localization methods

> "The battle of wits has begun," said the man in black. "It ends when you decide and we drink and find out who is right and who is dead."
>
> — *William Goldman, The Princess Bride*

Single-molecule localization methods are a subgroup of functional super-resolution and are defined by the basic common concept of separation and localization. Single-molecule localization microscopy is performed by manipulating the photochemistry so that only a small subset of the total fluorophore population is active (i.e. in the singlet cycle) at a given time. The sparsity of the active fluorophores isolates individual

emissions and allows the assumption of singularity, i.e. that every observed bright spot corresponds to exactly one fluorophore, and thereby allows to cheaply revert the PSF and localize the isolated fluorophore with subdiffraction precision. The most likely fluorophore position obtained by this procedure is known as a *localization*. After observing the fluorophore population for some time to ensure that most fluorophores have been in the active state at least once, the density distribution of the localizations is a super-resolved microscopy image of the sample. The plethora of super-resolution methods all share this basic concept and differ in the used fluorophore type and excitation scheme.

When fluorescent proteins are used, the terms PALM, FPALM and photoactivated localization microscopy with independently running acquisition (PALMIRA) are common. PALM and FPALM are synonymous and refer to microscopy on photoactivatable fluorescent proteins with alternating activation (ultraviolet light) and readout (visible light) phases. The term PALMIRA is used when activation and readout run concurrently.

Most other methods use synthetic organic dyes. The ancestor of the synthetic dye group is the STORM method that used pairs of different dyes with concurrent activation and recorded the FRET radiation. The *d*STORM, ground state depletion with individual molecule return (GSDIM), reversible photobleaching microscopy (RPM) and blink microscopy (BM) methods are synonyms and simplify STORM by using only a single dye instead of a pair. According to a recommendation by Baddeley [2011], I use the term *d*STORM in this work. In recent times, the original STORM method fell largely out of use in favor of *d*STORM, and some researchers [Jones et al., 2011] now use STORM as a synonym for *d*STORM.

The high-density problem

> Moonlight drowns out all but the brightest stars.
> — *J.R.R. Tolkien, The Lord of the Rings*

Single-molecule localization microscopy relies on the assumption that emitters are visible individually and resolved optically. This condition is somewhat fuzzy for two reasons: Firstly, while the term "resolved" is defined via the capacity of the human eye and a resolution criterion such as the Rayleigh resolution, it is unclear whether localization algorithms can reliably resolve all Rayleigh-resolved spots. Secondly, the single-molecule localization methods work stochastically and will produce optically unresolved molecules with an increasing probability at high spot densities. Therefore, it is not trivial to define where the high-density regime begins.

However, simple mathematics [Small, 2009] indicate that more than 10 diffraction limited regions of a common width, e.g. 300 nm, will hardly be resolvable when packed into a square micrometer. This density is easy to reach with photoswitching fluorophores when the ratio of the lifetimes of the dark (off) and the bright (on) state is insufficient. With a ratio of 100, less than 1000 molecules must be present in a square micrometer to reach even the very lax condition of 10 active fluorophores per square micrometer. This condition is in stark contrast to the Shannon-Nyquist sampling theorem [Shannon, 1984], which states that a signal (such as the presence or non-presence of a structure) must be sampled with at least twice the frequency at which it will be truthfully represented. Therefore, a continuous structure labelled in a perfectly equidistant pattern in our example would be resolvable to less than 60 nm.

1.4 Cellular biology

The cellular biology context for most super-resolved targets is well-known, and since I submit this thesis to a biological faculty, I assume familiarity with most of the concepts.

The discussion of the results of super-resolution microscopy depends strongly on the relevant biological sizes and scales. Many important cell organelles are below the diffraction limit, such as mitochondria with $\sim$ 100–500 nm width [Frey and Mannella, 2000], microtubuli $\sim$ with 50 nm [Heilemann et al., 2008] and the cell membrane with $\sim$ 10 nm. Many macromolecules have similar sizes, e.g. the ribosome with $\sim$ 20 nm and the RNA polymerase at $\sim$ 10 nm.

Optical super-resolution microscopy in the 20 nm domain can resolve the structure of small cell organelles [van de Linde et al., 2008] and map the spatial organization of the macromolecules, e.g. for the nuclear pore complex [Löschberger et al., 2012] or transcription factories [Cseresnyes et al., 2009].

Currently, the most common targets for super-resolution microscopy are the filamentous structures of actin or microtubulin. Both proteins have the advantage of being very common and forming the easily recognized cytoskeleton. The cytoskeleton's structure can be observed in a fluorescence microscope, but details remain unresolvable where individual filaments meet or overlap, thereby providing both a reference and a proof of resolution for super-resolution microscopy. Besides these technical points, the cytoskeleton has a considerable biological relevance as a key component of eukaryotic cells and as a factor in pathogen infection [Alberts et al., 2002].

Recently, critics [van de Linde et al., 2010] have shown that the filamentous nature of actin and microtubulin obscures the high-density problem (Section 1.3.1). A key non-filamentous target for super-resolution microscopy is the nuclear pore complex, which crosses the nuclear envelope membrane and provides transport through it. The nuclear pore complex's structure is well-documented through electron microscopy [Rout and Blobel, 1993] and both the ring macrostructure of 120 nm diameter and the eightfold-symmetric microstructure provide excellent observables for microscopy. As the main gateway for proteins and ribonucleic acid between the nucleus and the cytoplasm, the nuclear pore complex is of interest in many processes, and research into its dynamic behavior could yield very interesting scientific results.

1.5 Single-molecule localization algorithms

The core computational task of single-molecule localization microscopy is to find the position of a fluorophore (the *localization*) in a noisy camera image (the *spot*). Since a fluorophore may be approximated as point-like, its image on the camera is given by the PSF of the microscope as a function $\mathrm{PSF}(x, P, I)$ of the image plane position x, fluorophore position P and fluorophore intensity I. The image is distorted by noise from background signal, photon counting and readout noise effects [Mortensen et al., 2010].

Localization has been performed with one of three principal approaches:

- by building a functional model of the PSF and fitting it to the spot, or
- by using statistical properties of the spot, or
- by comparing the spot to previously taken calibration data.

The model-fitting approach has the advantages of a well-established theoretical framework and explicit modelling of defocusing effects, multi-plane setups and the various noise sources. It has the disadvantages of requiring a workable approximation of the PSF and of a high computational cost, since fitting algorithms are iterative and require one evaluation of the PSF per pixel and iteration step. The workable approximation of the PSF can be problematic because of the multitude of optical components in a modern microscope, which are almost impossible to model theoretically.

Statistical properties such as the center of mass are usually cheap to compute, but susceptible to noise. The separation of the localized fluorophore, other close-by fluorophores and background is difficult. Adapting statistical properties to the different PSFs found in dipole imaging or defocusing approaches is as complex as adapting a model function, since both approaches must prove the correctness of their approximations.

Comparing a spot to calibration data solves the approximation problem by measuring images of the real PSF on the same microscope at known offsets (*template* images) and then comparing each spot with the templates. This approach can easily deal with any form of PSF, but controlling noise is very difficult due to the lack of a theoretical model. Additionally, it is usually impossible to obtain and maintain a complete set of templates for each combination of spatial offsets, intensity differences and dipole orientation. Therefore, interpolation techniques must be employed to reduce the number of templates.

Early comparisons of localization algorithms have been made by Cheezum et al. [2001] and Thompson et al. [2002], who both arrived at the conclusion that fitting with a Gaussian PSF outperformed the simple statistical and template-based models of the time for 2D measurements. Later, Abraham et al. [2010] have extended the comparison on more precise PSF models in 3D, Mlodzianoski et al. [2009] have experimentally compared additional 3D PSF models, and Mortensen et al. [2010] have treated directional dipole imaging. A number of authors have advanced the PSF fitting algorithms with higher speed, 3D capabilities, and more precise PSF models [Thompson et al., 2002, 2010, Thomann et al., 2003, Ram et al., 2008, Starr et al., 2012, Aguet et al., 2005, Huang et al., 2008, Smith et al., 2010, Quan et al., 2010] or with approaches for high emitter densities [Holden et al., 2011, Huang et al., 2011, Babcock et al., 2012]. Statistical models were championed by Thompson et al. [2002], Hedde et al. [2009], Izeddin et al. [2012] and Ngo et al. [2012]. Baddeley et al. [2011] and Quirin et al. [2012] have improved the template-based methods beyond simple autocorrelation procedures.

1.5.1 The Gaussian point spread function model

Most researchers localize molecules by fitting a functional approximation of the PSF to the observed data. A functional approximation has the advantages that background, three-dimensional distortions or close-by fluorophores can be explicitly included in the model. However, the approach needs a functional model of the PSF that is both realistic and cheap to compute.

The Gaussian PSF with constant background is the most common choice and has been proven suitable for 2D and near-focal 3D imaging of molecules without a pronounced dipole orientation [Stallinga and Rieger, 2010, Zhang et al., 2007]. Its functional form for the image position (x, y, z), the fluorophore position (x_0, y_0, z_0), the fluorescence intensity I and the background intensity B is given in Eq. 1.2. The

PSF half-widths σ_x and σ_y are used to determine 3D behavior. For 2D imaging, suitable constants are chosen, and for 3D imaging they are functions of z and z_0.

$$G = B + \frac{I}{2\pi\sigma_x\sigma_y} \exp\left[-\frac{1}{2}\left(\frac{(x-x_0)^2}{\sigma_x^2} + \frac{(y-y_0)^2}{\sigma_y^2}\right)\right] \tag{1.2}$$

More precise models for the PSF have been researched by Ram et al. [2008] for very defocussed fluorophores and by Mortensen et al. [2010] for fixed dipole orientations.

The various noise effects cause uncertainty in the localization and prevent an exact localization. However, we can give a *likelihood* $\ell(S, E)$ of obtaining the spot S when observing an event E. An event is characterized by the parameters of the PSF, e.g. the variables in G for a Gaussian PSF. Accordingly, the objective of a localization algorithm is to find event that has the *maximum likelihood* among all possible events.

1.5.2 Least squares fitting and Levenberg-Marquardt

The difference between the model $G(x_i)$ and the data S_i is known as the *residue*. When we assume that the noise is normally distributed, the likelihood is maximal if and only if the squared sum of residues, i.e., the function χ^2_{LS} given by Eq. 1.3 is minimal. This assumption is reasonable in localization microscopy because hundreds of photons contribute to the photon-counting statistics in the center of the PSF and the side-lobes of the PSF have little overall contribution to the determined center position.

$$\chi^2_{\mathrm{LS}} = \frac{1}{\sigma^2}\sum_i \left[G(x_i) - S_i\right]^2 \tag{1.3}$$

The minimization problem is formalized by combining the unknown parameters of the PSF into a parameter vector a and considering χ^2 as a function of a. For nonlinear functions like the Gaussian PSF, there is no closed-form solution for the minima of $\chi^2(a)$. Therefore, the solution is determined iteratively by guessing an initial position a_0 and iteratively improving the position with the update equation $a_{i+1} = a_i + \delta_i$. Several iterative algorithms exist to provide values for δ_i.

The steepest descent method (Eq. 1.4) is the simplest solution. For every update step, we follow the negative gradient at the current position to obtain the next position. The length of the step is determined by a constant c. In practice, the choice of c is difficult, since high values overshoot the minimum and low values necessitate many update steps [Press et al., 1992].

$$\delta_i = -c\nabla\chi^2(a_i) \tag{1.4}$$

Newton's method avoids the constant by determining the minimum of the locally approximating paraboloid, which can be expressed as a linear equation system of the first and second derivatives (Eq. 1.5). Newton's method is parameter-free and converges quickly. However, Newton's method is prone to divergent oscillations [Press et al., 1992], and because the matrix of second-order derivatives is sometimes singular, Eq. 1.5 is not reliably solvable. The singularity problem is addressed by neglecting the second-order terms in the Hessian matrix $H(a)$ (Eq. 1.6). This approximation is valid

because the second-order terms are weighted by the residues. Because the residues close to a minimum are mostly caused by noise and therefore show little spatial correlation, they are equally likely to be positive or negative and their contributions cancel out. Since the reduced Hessian $\tilde{H}(a)$ is positive definite, Eq. 1.5 is always solvable.

$$
\begin{aligned}
H\delta_i &= -\nabla\chi^2(a_i) && (1.5)\\
H_{jk} &= \left(\nabla^2\chi^2\right)_{jk} && (1.6)\\
&= \sum_i \frac{1}{\sigma_i}\frac{\partial G}{\partial a_j}\frac{\partial G}{\partial a_k} - \sum_i \frac{(S_i - G)}{\sigma_i}\frac{\partial^2 G}{\partial a_j \partial a_k}\\
&\approx \sum_i \frac{1}{\sigma_i}\frac{\partial G}{\partial a_j}\frac{\partial G}{\partial a_k} = \tilde{H}_{jk}
\end{aligned}
$$

The Levenberg-Marquardt method combines the steepest descent and Newton's method [Press et al., 1992]. The diagonal elements of the reduced Hessian are multiplied by a factor of $\lambda + 1$, where λ is an adaptive parameter (Eq. 1.7). For low values of λ, Eq. 1.7 is identical to Newton's method with the reduced Hessian matrix. For high values of λ, the diagonal terms of the matrix dominate and the equation degenerates to steepest descent with $c = \lambda^{-1}$.

$$
\left[\tilde{H}(a_i) + \lambda \mathrm{diag}\left(\tilde{H}(a_i)\right)\right]\delta_i = -\nabla\chi^2(a_i) \qquad (1.7)
$$

The λ parameter is initialized with a pre-defined value, decreased whenever a step reduced the value of χ^2, and increased whenever the computed step would increase the residues. Thereby, Newton's method is preferred when the function is well-behaved, and the more reliable steepest descent method is preferred with increasingly shorter steps when the function is ill-behaved.

The implementation chooses criteria for stopping the iteration. Usually, relative or absolute thresholds for the shift vector δ_i, the remaining value of χ^2 or an absolute limit on the number of steps are used. The initial guess a_0 is provided by using elementary statistics on the spot. The choice of initial values is an important influence on the performance of the fitting algorithm.

1.5.3 Maximum-likelihood fitting

A statistical estimator is termed a maximum likelihood estimator (MLE) when its estimated quantities have maximal likelihood. In other terms, when a MLE estimates a set of model parameters, there should be no other choice of the model parameters that yields a higher probability of producing the observed data.

Least-squares fitting is a MLE when the noise is normally distributed. While this is a fairly close approximation for the very intense center pixels of the bright emission of a good fluorophore in focus, the approximation is insufficient for low photon yields, smeared-out spots in 3D, or dipole-directed imaging [Mortensen et al., 2010]. Even for classic localization microscopy, the peripheral pixels of a spot receive few photons, therefore show Poissonian instead of Gaussian noise distribution, are inaccurately accounted by least-squares algorithms, and therefore can contribute to more precise localizations with a MLE method.

Using MLE for Poissonian statistics can be implemented with the reliable Levenberg-Marquardt method [Laurence and Chromy, 2010] by weighting the differences between model function and measured data according to Eq. 1.8 when both model and measured data are given in photons. However, the use of a Poissonian MLE requires the additional parametrization of camera sensitivity and offset to compute estimated photon counts, and computational effort is higher than least-squares estimation [Laurence and Chromy, 2010].

$$\chi^2_{\mathrm{MLE}} = 2\sum_i G(x_i) - S_i\left(1 + \ln\frac{G_i}{S_i}\right) \tag{1.8}$$

1.6 Software development

> Science is what we understand well enough to explain to a computer.
> Art is everything else we do.
>
> — *Donald Knuth, Foreword to the book A = B*

Software development for single-molecule localization software faces three critical issues. Firstly, the field is far from stable, and the requirements for data evaluation change daily and often in response to the advances made in the software itself. Secondly, the field is quickly expanding, and the simple core localization algorithm must be made available in a great range of situations, ranging from traditional, well-understood two-dimensional (2D) *d*STORM measurements to extremely complex and little-understood scenarios with nine different optical paths [Hajj, 2012]. The former experiments require fire-and-forget software that can be used with minimal training and expertise, while the latter experiments necessitate a great deal of control and diagnostic on the software. Thirdly, performance is an issue, since millions of localizations must be computed and the number of variable parameters in experiment and evaluation makes short feedback loops essential.

1.6.1 Prior and concurrent art

Prior and concurrent art to this thesis has been published mostly by the scientific community. From the one direction, experimental groups have established a number of ad-hoc scripts and ImageJ plugins, such as QuickPALM [Henriques et al., 2010], Palm3D [York et al., 2011], FluoroBancroft [Hedde et al., 2009], Octane [Niu and Yu, 2008], or Thomann's pioneer work [Thomann et al., 2002]. From the other direction, theoretical groups have scrutinized and extended the theoretical limits of localization microscopy and produced tools and algorithms such as MUMLA [Ram et al., 2008] used in EstimationTool [Abraham et al., 2010], Mortensen's maximum-likelihood fitting [Mortensen et al., 2010], Gaussian mask fitting [Thompson et al., 2002], M^2*L*E [Starr et al., 2012], DAOSTORM [Holden et al., 2011], Izeddin's wavelet localization [Izeddin et al., 2012], Huang's GPU multi-emitter fitting [Huang et al., 2011] or Zhu's compressed sensing approach [Zhu et al., 2012].

All of these projects have limited utility in the biological laboratory since they lacked the scope to comprehensively solve the localization task or were intended as a proof of concept. From the group of theoretically-motivated tools, only EstimationTool, DAOSTORM and M^{2}LE were released to the public, and only the EstimationTool has made it past an early prototype stage. The rest of the projects have been

abandoned shortly after the initial release according to their public code repositories, since their scientific usefulness to the original authors had ended after the feasibility of the algorithm had been tested. The experimentally-motivated software projects have all suffered under their side-project natures and have received neither the time nor the expertise necessary to implement high-precision localization algorithms that approach or surpass the precision of least-squares Gaussian fitting. Due to the extreme speed of advances in localization microscopy, the maintainer of a local microscopy setup is unable to keep both the physical setup and the software maintained, and the latter slowly obsoletes.

1.6.2 Free, open source and open access software

The terms *free software* and *open source software* are both well defined and different in meaning, and they should be contrasted to the scientific term *open access*. The term of *free software* was defined by the Free Software Foundation as Stallman [2002]

> software that respects users' freedom and community. Roughly, the users have the freedom to run, copy, distribute, study, change and improve the software. With these freedoms, the users (both individually and collectively) control the program and what it does for them.

The term is distinguished from *open source software* by the Open Source Initiative, which defines open source software as software where the source code is freely available, free from royalties or restrictive licenses [Perens, 1999].

The essential difference, as described by Stallman [2009], is the focus of *free software* on the user's freedom of changing, adapting and controlling his software, while *open source software* focuses on the chance to examine, modify and redistribute the software source code. While the difference have been mostly academic in the past and the categories of free and open-source mostly overlap, free and open-source software diverge on the practice of "tivoization" [Stallman, 2009], i.e. software-controlled devices that do not allow the user to install his own software such as Android-controlled smart phones. Tivoized software is never free, but can be open-source.

The software developed in this thesis is both free and open software. The scientific merits of open-source software are immediately clear and, in my view, indispensable to reproducible and traceable computational science: When research advances are driven largely by software, the exact details of the algorithm and its implementation are essential to understanding and interpreting the results. The methodical section of the scientific article must contain at least pseudocode about the computational part, as a multitude of hardly reproducible articles about single-molecule localization strategies shows. Textual descriptions of algorithms are always incomplete and lack essential detail. However, the task of converting real code to pseudocode is both lengthy and error-prone, and should be avoided. Therefore, I pushed for rapid, accurate program implementing direct stochastic optical reconstruction microscopy (rapi*d*STORM) to be open-source software in the interest of the scientific principle.

The benefit of free software to the community is also clear: When researchers can freely copy, modify and use the essential software on their field, software-driven methods become truly open and are easily reproduced, reviewed and improved throughout the scientific community. The merits of this process have become clear for traditional experimental methods and protocols in the scientific revolution, and should be applied to software as well.

Free and open-source software must be contrasted against the *open access* paradigm. *Open access* is a movement that tries to free scientific writing of the ever-increasing burden of the subscription-fee-heavy scientific journal. Open access articles may be viewed in full text at no cost to the reader, but the open access confers no right to derivative work. As such, open access is a much weaker principle than free or open source. However, it is well suited to the realities of scientific research, since research papers are made to be *understood*, not to be *executed*, and because the typical research paper has 500 lines of text rather than 50,000 like a reasonably complete software system. When the ideas in a research paper need to be re-used, a partial rewrite can be done with a long afternoon and a bottle of wine; for research software, a semester and a barrel of rum are a low estimate.

1.6.3 Agile development

> I've found from past experiences that the tighter your plan, the more likely you are to run into something unpredictable.
>
> — *MacGyver, The Heist*

The speed of change in localization microscopy poses a challenge to traditional software development methods. Waterfall development, i.e. a single sequence of the stages of specification, design, implementation, testing and deployment of software, is impossible to implement because any sufficiently complete and usable software is obsolete before it is deployed.

This problem is common in software development and a core question of the discipline of *software engineering*. However, many established solutions such as Scrum [Schwaber, 2004] or Kanban [Epping, 2011] focus on the software development team and the industrial process, with only few lessons applicable to the scientific environment.

The work of Martin [2003] and Beck [2002] is a notable exception because of its focus on refactoring and automated testing. By establishing a test suite that covers the program's functionality, safe changes to the program structure (*refactoring*) become possible. Through the speed of automated tests, the cost of releasing tested software becomes small, and a quick release cycle can be adopted safely. Since users always hold a current and usable version, the vital cycle between usage feedback and program design and development is kept so short that continuous software improvement is possible.

Chapter 2

Articles

This chapter contains the most important articles that I have written in my thesis. The articles appear as they were typeset by the publishing journal. Every article is followed by a related declaration of independent work that states, in German language, my share of the work in the article.

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Chapter 3

Discussion

3.1 Software creation

The scientific advance due to the rapi*d*STORM project lies in two aspects, the simplification and the publication of the data evaluation for single-molecule localization microscopy. Section 2.1 was the first published real-time localization software for full least-squares fitting with a Gaussian function. At the time, the community considered least-squares Gaussian fitting as a very precise, but computationally too expensive method, and searched heavily for alternatives that would provide cheaper real-time analysis [Thompson et al., 2002, Hedde et al., 2009]. Section 2.1 proved that least-squares Gaussian fitting is real-time capable with a comfortable speed margin, and gave a concise description of the algorithm. However, it did not provide a publicly available program, and it did not show any three-dimensional or multi-color algorithms. Therefore, its primary value to the scientific community was the demonstration of the potential speed of Gaussian fitting. While the highly precise maximum-likelihood formulations of super-resolution microscopy had been known for years [Aguet et al., 2005], late 2010 and 2011 saw a much refreshed interest in highly precise super-resolution microscopy [Mortensen et al., 2010, Quan et al., 2010]. As such, the work by Wolter et al. [2010] considerably advanced the field.

In the following years, rapi*d*STORM was constantly expanded in functionality, released to the public by Wolter and Sauer [2012] (Section 2.3), and its use on a breadth of multi-color and 3D applications was demonstrated by Wolter et al. [2012a] (Section 2.4). The second article marked the first time that broadly applicable, mature super-resolution software made all algorithmic details available as open-source software, and the scientific community has recognized this fact by downloading the rapi*d*STORM software from over 200 second-level domains (as of early 2013, see Chapter D). The accumulated knowledge was integrated into textbooks by Wolter et al. [2014] (Section 2.6).

I primarily spent the two years between the first and the last rapi*d*STORM publication on the rapi*d*STORM project. rapi*d*STORM went through two major revisions as the design evolved, and saw a considerable number of succeeding and failing interface experiments, platform changes, refactoring, and technological issues. rapi*d*STORM is still the most extensive and a very fast solution for localization microscopy [Wolter et al., 2012a]. It currently has around 60,000 lines of code with low duplication, roughly comparable to the UNIX operating system version 6 Etsion et al.

[2007], which was one of the first publicly available operating systems and ran the majority of academia's computers.[1]

From the viewpoints of software engineering, the rapi*d*STORM project was successful. The application of continuous integration, test-driven development and release-early-release-often to rapi*d*STORM was successful. In the year of 2012, rapi*d*STORM went through 15 public releases, more than one per month on average and five per month, a one-week cycle, in busy times. Currently, 110 use cases and regressions are under test, with an estimated code coverage of 60%. The project was able to survive a complete reimplementation of the core fitting function with minimal branching.

However, one of the core goals of open-source projects, a wide and self-supporting user community that participates in development, could not be reached. I explain this failure by three reasons, prioritized by estimated impact:

1. The choice of the implementation language C++ has been motivated technically without taking the skills of the wider community into account. Computer literacy in the biological community is focused on scripting languages like Matlab or Python and may include memory-managed object-oriented languages at best. C++ combines the considerable complexities of explicit memory management, object orientation and its unique template mechanism. While all of these features have played important roles in the success of rapi*d*STORM, they also complicate the search for codevelopers and maintainers greatly. In the future, the rapi*d*STORM project will have to find ways to interoperate with the non-C++ community.

2. Public releases had to be unified with the demands of the scientific publishers. The editorial boards of important journals such as Nature Methods consider open-source software as prior publication. Consequently, important advances needed to await the lengthy board-review and peer-review processes.

3. Inter-group competition in single-molecule localization microscopy is intense. The principal drivers of the three major single-molecule localization microscopy methods Eric Betzig (PALM), Xiaowei Zhuang (STORM) and Markus Sauer (dSTORM) have not co-authored a single paper. In this view, the close association of the rapi*d*STORM project with the Sauer group has been a mixed blessing; while the group has given invaluable support to the project[2], it has also tied the project into its sphere of influence, and the intense atmosphere of competition in single-molecule localization microscopy has prevented wider cooperation.

3.2 Three-dimensional evaluation

One of the important future challenges of localization microscopy will be the definition and verification of widely usable and reliable 3D protocols. Despite large interest in 3D microscopy, and a number of high-profile articles in the mid-2000s [Holtzer et al., 2007,

[1] Admittedly, in today's numbers, this would be a classroom full of antiques that even undergraduates wouldn't touch with a 10 foot pole. I assume that the majority of the thesis examination committee was young or very young back then, and I'd like to invite you to indulge in nostalgia. Having a good feeling during grading time makes all of us happy.

[2] Please allow me to re-iterate the acknowledgements at this point: I thank the Sauer sincerely and most gratefully for their unfailing, most helpful support and friendly atmosphere.

Huang et al., 2008], 3D calibration methods have not been systematically compared against ground truth data and each other. Even commercial products show large inconsistencies in results [Proppert et al., 2014, Section 2.5]. Most researchers stop at measuring the precision of new methods by determining the half-width of filaments or other structures. Inaccurate lateral and axial measurements are not covered by this test in any way, but cause wrong results for distance and speed measurements.

The problem did not surface in 2D microscopy due to the high reliability of two-dimensional optics, and was not noticed earlier in 3D microscopy since most laboratories roll their own 3D solutions, give very little detail in publications and therefore make reproduction of experiments prohibitively expensive, if not outright impossible.

The publication of full source code along with operational procedures is a scientific necessity, and rapi*d*STORM's open source nature has taken an important step. Even more important, in Proppert et al. [2014, Section 2.5] I have greatly simplified the calibration process, which is a necessary first step towards weeding out bad algorithms instead of hunting for bad parameter choices. Cubic B-splines are a natural choice for the interpolation and smoothing of the noisy 3D calibration data, and my coauthors have demonstrated the feasibility in the laboratory process excellently.

3.3 Multi-color and multi-plane evaluation

rapi*d*STORM is still one of the few software solutions that offers support for biplane 3D and multi-color evaluation. Both approaches are impeded by the computational need to aggregate the pixel data of multiple cameras, while dealing with optical misalignment and different fluorophore types. Available software is either theoretically motivated and slow [Ram et al., 2008], or is a postprocessing step that collects localization data from independent evaluation of the two planes and gains only minimal information from the cross-talk [Lampe et al., 2012]. Multifocus optics [Abrahamsson et al., 2013] can increase the number of planes to nine, which is computable only by very few software packages, including rapi*d*STORM [Hajj, 2012].

I strongly suspect that this relative lack of software support is due to the aggravation of the overfitting effect that many researchers ignore in 2D evaluation. The 2D Gaussian has 5 free parameters: amplitude, position in X and Y, and width in X and Y. The width in X and Y is a function of the optics and does not vary for each spot, but is still fitted individually to every spot by most softwares. When doing multi-color fitting in two planes, the gap between 3 necessary free parameters and 5 possible free parameters widens to 3 necessary free parameters (fluorophore brightness, position in X and Y) against 10 possible free parameters. The overfitting with 10 free parameters is causing intense problems, and poses an unacceptable barrier for ad-hoc development.

In rapi*d*STORM and Wolter et al. [2012a], I have demonstrated how the 3 necessary free PSF parameters can be inserted into the PSF as explicit and mutually independent parameters. This work is mathematical and adaptable to other software, and I am confident that it will make multi-plane fitting much more reliable and widespread.

3.4 High-density problem

van de Linde et al. [2010, Section 2.7] and Wolter et al. [2011, Section 2.8] defined,

motivated and characterized the high-density problem of single-molecule localization microscopy. To summarize, I have shown in these articles that classical single-molecule localization algorithms quickly lose both precision and accuracy in spot identification when too many molecules are concurrently active in the sample. Since the control over the number of active molecules is not complete, this can pose a problem for single-molecule localization microscopy on some samples.

I found that a realistic limit of concurrently active fluorophores is 0.6 molecules per square micrometer. This limit not only places restrictions on the necessary level of photophysical emitter control, but also defines a lower limit for the acquisition time and thereby the observation of dynamic processes.

A number of algorithms capable of processing higher-density data has been suggested while this work was performed. Both multi-molecule [Holden et al., 2011, Huang et al., 2011, Babcock et al., 2012] and true ensemble [Dertinger et al., 2009, Zhu et al., 2012, Cox et al., 2011] techniques have been established and tested. The primary contribution of my work is the development and demonstration of methods to quickly and reliably measure the performance of localization algorithms with the three core observables of

1. spatial localization precision, i.e. the average spatial error in localization,

2. statistical precision, i.e. the rate of false positives in localization, and

3. statistical accuracy, i.e. the rate of false negatives in localization.

These three observables are critical to characterizing, comparing and observing algorithms. Some authors combine statistical precision and accuracy into the F-measure [Křížek et al., 2011], but most authors focus only on localization precision and do not measure or characterize stochastic precision. So far, the improved measurement methods proposed by [Wolter et al., 2011, Section 2.8] have failed to gain widespread use in the field.

Consequently, the lasting contribution of these articles is the precise characterization of the widely used Gaussian-PSF single-kernel fit approach and the moderate and simple improve in high-density performance gained with second-kernel checking.

3.5 Biological applications

Sections 2.9, 2.10 and 2.11 document my efforts to push single-molecule localization microscopy into the dynamical domain. The ability to capture dynamic, real-world biological processes will determine the role of super-resolution microscopy in biology by giving super-resolution fluorescence microscopy a functional edge over electron microscopy. The electron microscope can be used for almost all static applications where single-molecule localization microscopy is useful, but its high staining requirements prevent the imaging of dynamic processes.

Endesfelder et al. [2010, Section 2.9] have shown how single-molecule localization microscopy can be used to detect and track motion. I contributed the color-coded sliding window visualization that made movement visible in much better context than traditional video visualization. The findings of Endesfelder et al. [2010] are of particular importance to the method because they eliminate the common concern that single-molecule localization would be too slow to address relevant biological questions.

We have learned from these myosin-actin motility experiments that *d*STORM is sufficiently fast for certain dynamic biological processes, but also learned that the error margin is not particularly large and that slower imaging methods, e.g. PALM, would have been insufficient to conduct the experiments.

Klein et al. [2011, Section 2.10] have demonstrated the live-cell capability of *d*STORM, and thereby clarified whether super-resolution with synthetic dyes could be performed in a cell environment. While my role in the research was mostly advisory, I included this article in the thesis to stress its importance to super-resolution microscopy. The question of intracellular super-resolution use of synthetic dyes gains special relevance when combined with the results of sections 2.1 and 2.9: *d*STORM imaging has a strong advantage over both fluorescent proteins and electron microscopy when imaging fast-moving processes, but since a process can only be meaningfully monitored within the cell, methods for intracellular imaging are crucial for the success of *d*STORM and localization microscopy in general.

When live monitoring of dynamic processes is unavailable, researchers must substitute a sequence of snapshots. Bar-On et al. [2012, Section 2.11] have studied the dynamic process of syntaxin clustering. This study is full of highly novel statistical methods, and I contributed the idea and implementation of finding clusters of localizations by proximity to the local maximum in a smoothed density image. Both the number of statistical methods and the length of scientific study from the first grant 2007 to the publication of results in 2012 shows the difficulty of using snapshots to analyze complex dynamic processes. The methods once more underline the importance of our ability to observe and evaluate dynamical processes in the cell.

Section 2.10 also shows the difficulties and problems associated with strong and inhomogeneous background in localization microscopy. After the publication of the article [Klein et al., 2011] I have addressed the problem in software with the introduction of a band-pass spot detection method and background-relative spot thresholding methods [Wolter et al., 2012b]. While rigorous, peer-reviewed performance measurements are still outstanding, the method has shown promising results on data sets with high and inhomogeneous background.

Chapter 4

Conclusion and Outlook

In this thesis, I have proven that single-molecule localization microscopy evaluation software can be fast, flexible, versatile and precise at once. I have implemented and documented a free, open-source software. The result is widely used and brought single-molecule localization microscopy considerably closer to widespread use in the biological laboratory.

I have demonstrated that the various branches and new techniques of single-molecule localization microscopy can be united in a single evaluation software and a single localization algorithm. Very precise maximum-likelihood estimation localization, multi-plane microscopy and three-dimensional imaging were combined in rapi*d*STORM with a single localization algorithm based on the well-established Gaussian PSF, yielding an excellently tested and reliable implementation as a base for future method research.

The fast, free and quickly installed rapi*d*STORM software advances work in the biological laboratory in three ways:

1. deployment costs for single-molecule localization imaging are decimated by the ease of use, open access, wide platform compatibility and low dependencies,

2. transparency and reproducability of scientific protocols are guaranteed by allowing access to versioned, reliably published and independently archived software, and

3. the open-sourced code provides complete and human-readable documentation of the algorithmic procedures.

I have characterized the performance of the Gaussian least-squares localization method with respect to the high-density problem, and shown a low-cost way to improve the performance using the two-kernel improvement. The development of a method to measure spatial localization performance, recognition precision and recognition accuracy independently of each other has been an important part of this work. I have located the limit of sensible single-molecule localization microscopy with classic localization algorithms to 0.6 concurrently active molecules per squared micrometer.

I have greatly simplified calibration for three-dimensional localization by introducing, testing and characterizing cubic B-splines for describing the relationship between axial position and width of the Gaussian PSF. The model offers both high accuracy and high precision, combining the best of previous approaches.

I have applied and extended single-molecule localization microscopy towards the critical goal of visualizing dynamic processes in the living cell with super-resolution fluorescence microscopy. To that end, I have developed software methods to study myosin-actin motility *in vitro*, to analyze live-cell *d*STORM measurements, and to gain understanding about the dynamic processes of syntaxin clusters from static snapshots of the membrane.

The most lasting impact and greatest challenge for future work is the availability of sufficiently complete open source software for single-molecule localization microscopy. The current challenge for the field and the rapi*d*STORM project is the lack of a vibrant, connected community to bundle and publish efforts, mostly for reasons of technical complexity and lack of inter-lab communication. This challenge needs to be overcome to make single-molecule localization microscopy a widely accepted staple technique for microscopy.

Even though a host of high-density localization algorithms have been presented, a rigorous comparison is still lacking. Most papers in the field do not give all three relevant statistics of localization precision, recall and stochastic precision, or have deficient measurement procedures.

Clear physical proof of the accuracy of three-dimensional localization microscopy is desperately needed. The current literature contains only proof of the precision by measuring the half-width of obtained distributions, but accuracy is critical to determining distances or speeds in the sample. While our method has shown internal consistency, a reference sample is needed to prove the correctness of the procedure.

Appendix A

Ehrenwörtliche Erklärung

Hiermit versichere ich an Eides statt, dass ich die Dissertation selbständig angefertigt habe. Übernommene Inhalte wurden von mir eindeutig gekennzeichnet.

Die Gelegenheit zum Promotionsverfahren wurde mir nicht kommerziell vermittelt. Insbesondere wurde keine Person oder Organisation eingeschaltet, die gegen Entgelt Betreuer bzw. Betreuerinnen für die Anfertigung von Dissertationen sucht.

Ich erkläre weiterhin, dass ich die Regeln der Universität Würzburg über gute wissenschaftliche Praxis eingehalten habe.

Meine Dissertation wurde weder vollständig noch teilweise schon einmal einer anderen Fakultät mit dem Ziel, einen akademischen Grad zu erzielen, vorgelegt. Teile dieser kumulativen Arbeit wurden publiziert und werden hier mit der Erlaubnis der einzelnen Zeitschriften verwendet bzw. reproduziert.

Mir wurde der akademische Grad des Diplom-Informatikers durch die Universität Bielefeld verliehen. Ich habe bis zum heutigen Tag keinen anderen akademischen Grad erworben, noch versucht solche zu erwerben.

Es wurde mir kein akademischer Grad entzogen, noch wurde gegen mich diesbezüglich ein strafrechtliches Ermittlungsverfahren oder Disziplinarverfahren eingeleitet.

München, September 2014

Steve Wolter

Appendix B

Acknowledgements

More than ever, today's science is a collaborative endeavor. Single-molecule localization microscopy as a field would be nonexistent without a healthy dose of interdisciplinarity. This thesis depended on the support of the Sauer research group, who went out of their way to provide experimental data and verification for the development of rapi*d*STORM. Special thanks goes to Markus Sauer, who never wavered under the quintuple workload of marketing, management, requirements engineering, advisor and mentor, and always found time where time was needed; to Sebastian van de Linde, who drove the development of *d*STORM with élan and passion; to Mark Schüttpelz, who pioneered localization microscopy evaluation in the Sauer group in Bielefeld; to Mike Heilemann, who gave great support and direction in the early stages; to Stefan Bollmann, Sven Proppert, Thomas Niehörster and Simon Hennig, whose support, discussions and collegiality made my office a happier place; to my bachelor students Timo Bressmer and Richard Pfaller, whose work helped to identify dead ends in the preparation of this thesis; and last but not least to Thorge Holm, Teresa Klein, Anna Löschberger and Ulrike Endesfelder for their valor in preparing the excellent raw data you can see in this thesis. I thank all members of the Sauer and Heilemann groups for the outstandingly excellent and constructive atmosphere: It was a pleasure to work with every one of them. I thank the Bundesministerium für Bildung und Forschung (BMBF) and the Deutsche Forschungsgemeinschaft (DFG) for their financial support, and the Acme Packet company for their patient understanding of the final phase of my work.

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Appendix C

Acronyms

2D	two-dimensional ... 16
3B	bayesian analysis of blinking and bleaching ... 10
4PI	4Pi microscopy ... 8
AWESOME	AWESOME wide-field excitation super-resolution optical microscopy except SOFI ... 10
BM	blink microscopy ... 11
BMBF	Bundesministerium für Bildung und Forschung ... 181
CAFM	critical angle fluorescence microscopy ... 4
DFG	Deutsche Forschungsgemeinschaft ... 181
dSTORM	direct stochastic optical reconstruction microscopy ... 10
FPALM	fluorescence photoactivation localization microscopy ... 10
FRET	Förster resonance energy transfer ... 8
GFP	green fluorescent protein ... 1
GSD	ground-state depletion ... 8
GSDIM	ground state depletion with individual molecule return ... 11
HILO	highly inclined and laminated optical sheet ... 4
LAO	low-angle oblique ... 4
MLE	maximum likelihood estimator ... 15
NSOM	near-field scanning optical microscopy ... 8
PALMIRA	photoactivated localization microscopy with independently running acquisition ... 11
PALM	photoactivated localization microscopy ... 10
PET	photoinduced electron transfer ... 8
PSF	point spread function ... v
rapidSTORM	rapid, accurate program implementing direct stochastic optical reconstruction microscopy ... 17
RESOLFT	reversible saturable optical flurorescence transitions ... 10

RPM reversible photobleaching microscopy 11
SIM structured illumination 1
SOFI super-resolution optical fluctuation imaging 1
SPDM spectral precision distance microscopy 10
SSIM saturated structured illumination microscopy 8
STED stimulated emission depletion 8
STORM stochastic optical reconstruction microscopy 10
TIR total internal reflection 3

Appendix D

rapidSTORM downloads

This section lists the 219 second-level domains that initiated downloads of rapi*d*STORM. The domain name component of `ac` was ignored in counting domain levels due to its frequent use as an university qualificator in domain names. It can be reasonably expected that each of these domain names corresponds to at least one research group, with the effects of multiple groups per domain and researchers accessing rapi*d*STORM from multiple domains (e.g. home and work) cancelling out. To protect the privacy of the involved people, access dates and IP addresses are not listed and available upon request.

above.net	alicedsl.de	amc.nl	amres.ac.rs
arcor-ip.net	auckland.ac.nz	azn.nl	bbtec.net
bell.ca	Berkeley.edu	bionand.es	bluewin.ch
broadviewnet.net	btcentralplus.com	buffalo.edu	cablecom.ch
cableone.net	caltech.edu	cam.ac.uk	cancerresearchuk.org
cas.cz	cchmc.org	cea.fr	chalmers.se
charter.com	cmu.edu	cnrs.fr	cnrs-gif.fr
co.in	co.jp	colorado.edu	com.ar
com.au	comcast.net	com.cn	com.sg
cornell.edu	co.uk	cox.net	cuni.cz
dkfz-heidelberg.de	dote.hu	ed.ac.uk	edu.au
edu.sg	edu.tr	edu.tw	ehu.es
embl.de	ens.fr	ens-lyon.fr	epfl.ch
erasmusmc.nl	ethz.ch	everestkc.net	feico.com
fmp-berlin.de	fraunhofer.de	frontiernet.net	fu-berlin.de
gla.ac.uk	globonet.hu	googlebot.com	gulbenkian.pt
gwdg.de	hamamatsu.ch	harvard.edu	helsinki.fi
hwk-ufr.de	ibl.fr	ic.ac.uk	ifn-magdeburg.de
indiana.edu	ipht-jena.de	jhmi.edu	kabel-badenwuerttemberg.de
kabsi.at	kfa-juelich.de	kit.edu	ku.dk
kuleuven.be	kyoto-u.ac.jp	lanl.gov	lbl.gov
masterplanet.fi	mcgill.ca	mcmaster.ca	mediaways.net
meduniwien.ac.at	mh-hannover.de	mit.edu	mnet-online.de
m-online.net	mpg.de	mrc.ac.uk	mts.ru
mtu-net.ru	muni.cz	mwn.de	net.au
net.br	net.il	netvigator.com	nki.nl

numericable.fr
nyu.edu
oaps.eu
oist.jp
online.nl
optonline.net
ox.ac.uk
pasteur.fr
pnl.gov
princeton.edu
proxad.net
purdue.edu
qmul.ac.uk
rice.edu
rl.ac.uk
rogers.com
rr.com
rug.nl
rwth-aachen.de
salk.edu
sbcglobal.net
scansafe.net
scripps.edu
sfr.net
shef.ac.uk
spectral.ca
stjude.org
sunysb.edu
superkabel.de
susx.ac.uk
swmed.edu
swmed.org
tamhsc.edu
tau.ac.il
t-dialin.net
telekom.hu
teleweb.at
t-ipconnect.de
tohoku.ac.jp
tropolys.de
tu-berlin.de
tu-bs.de
tu-darmstadt.de
tudelft.net
tuwien.ac.at
twtelecom.net
u-3mrs.fr
u-bordeaux2.fr
u-bordeaux.fr
uchicago.edu
uclm.es
ucsd.edu
ucsf.edu
uga.edu
uio.no
uiuc.edu
uk-erlangen.de
umass.edu
umds.ac.uk
umich.edu
uniba.it
uni-bielefeld.de
uni-bonn.de
uni-erlangen.de
uni-frankfurt.de
uni-freiburg.de
uni-goettingen.de
uni-hamburg.de
uni-hannover.de
uni-heidelberg.de
uni-jena.de
uni-karlsruhe.de
uni-kassel.de
uni-konstanz.de
unilever.com
uni-magdeburg.de
uni-mainz.de
uni-muenster.de
unipd.it
uni-regensburg.de
uni-siegen.de
uni-stuttgart.de
univ-mrs.fr
uni-wuerzburg.de
uoc.gr
uoregon.edu
upc.es
u-psud.fr
usc.edu
u-strasbg.fr
u-tokyo.ac.jp
utu.fi
utwente.nl
uu.nl
uu.se
uva.nl
verizon.net
versatel.nl
videotron.ca
virginmedia.com
vtt.fi
vu.nl
wanadoo.fr
wisc.edu
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wustl.edu
xs4all.nl
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