Fabrication of Directly Functionalized Polymer Structures using STED Lithography

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Linz, August 2015
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Abstract

This diploma thesis describes a method for fabricating functionalized polymer structures by multiphoton polymerization in combination with stimulated emission depletion (STED) lithography. This is achieved by simply adding metal oxo clusters to the negative tone photoresist. The clusters carry two functionalities, methacrylates used within the polymerization process and mercapto groups. The mercapto ligands stay intact and accessible and serve as binding sites after the polymerization process.

The minimum feature size of the structures is about 100 nm when using multiphoton polymerization (@ 780 nm). Introduction of a donut-shaped depletion beam (@ 532 nm) leads to a 28% restriction of the line width resulting in feature sizes of about 70 nm.

Using Alexa647-maleimide for incubation of the mercapto functionalized structures allows for investigation of the structure functionalization. The maleimide side of the linker binds covalently to the accessible mercapto groups, while the Alexa647 signal is detected using confocal laser scanning microscopy (LSM). Structures containing functionalized clusters show a thirtyfold increased fluorescence signal intensity compared to structures without mercapto groups.
Zusammenfassung


Durch Multiphotonenpolymerisation (@ 780 nm) können Strukturen mit einer minimalen Größe von etwa 100 nm hergestellt werden. Bei zusätzlicher Verwendung eines ringförmigen Abregestrahls (@ 532 nm) kann die Linienbreite der Strukturen um 28% verringert werden. Dies entspricht Linienbreiten von ca. 70 nm.

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1 Introduction

Modern light microscopy enables to observe and gather spatial information in a range of less than 10 nm in three dimensions [1]. Labeling of samples with small, emitting molecules called fluorophores makes it possible to image living organisms in three dimensions in their natural environment. Moreover, observation of dynamic processes in living cells is possible, which is another advantage over other microscopy techniques. This makes fluorescence microscopy to the most important microscopy technique in biology and life sciences. However, the resolution in light microscopy is diffraction limited. Thus the numeric aperture (NA) as well as the light wavelength determines the resolution, which happens to be a weakness over microscopy techniques like atomic force microscopy. In 1994, Stefan W. Hell introduced a method to overcome this resolution limit [2]. The invention of stimulated emission depletion (STED) microscopy was recently honored with the Nobel Prize in Chemistry. The basic idea of STED microscopy is to reduce the effective excitation volume (point spread function – PSF). This is achieved by de-excitation of fluorophores in the outer rim of the excitation volume. The switching of fluorescent molecules happens due to stimulated emission.

Optical lithography is often referred to as the opposite mechanism to light microscopy. A material known as photoresist contains small initiating molecules. Those molecules get excited and initiate a chemical reaction, which leads to a solubility change of the photoresist. Using two photon polymerization (TPP) enables to fabricate nanostructures even in three dimensions [3]. These techniques are, similar to microscopy techniques, diffraction limited. Therefore the challenges, which appear for lithography and microscopy, are comparable. To achieve features below the diffraction limit STED lithography is used. This method enables structuring with a feature size of 55 nm and a resolution of 120 nm [4].

The nanostructures fabricated with optical lithography are useful in a great variety of different research fields reaching from optical metamaterials to biological applications [5–7]. However, it is of growing interest to fabricate micro- and nanostructures with functional groups. Upon doping of the basis photoresist with clusters allows for fabricating polymer structures with different properties. The presented diploma thesis describes a method for directly functionalizing polymer structures with mercapto groups. Therefore low amounts of functional metal clusters are used for doping of the photoresist.
Those metal clusters (cluster core consisting of zirconium and titan-zirconium, respectively) are bifunctional as they have both, methacrylates and mercapto groups, as ligands. The amount of mercapto groups on each cluster molecule can be varied. Mixing of those clusters into the photoresist allows for fabricating mercapto functionalized polymer structures. A 28% reduction in structure size can be realized using STED lithography (about 70 nm feature size using STED).

The presented diploma thesis is divided into the following chapters: Chapter 2 gives an introduction to fluorescence microscopy and the super-resolution methods STED microscopy and laser scanning microscopy (LSM). The related method optical lithography, including STED lithography is the topic of chapter 3. Special interest is laid on the different photoresists, i.e. monomers and photo-initiators, available for STED lithography. Chapter 4 will introduce the experimental setups and materials used for this diploma thesis. Moreover, a description of the sample preparation process and the experiments will be given. The results of the experiments will be presented and discussed in chapter 5. Finally, chapter 6 gives a summary of this diploma thesis.
2 Fluorescence Microscopy

With the invention of the light microscope, a new era, especially in life sciences, has started. Using early light microscopes enabled to observe cells as an important feature of plant tissue. With the invention of fluorescence microscopy, the investigation of biological samples in a non-invasive way and in their natural environment became possible. Sample labeling with small molecules, called fluorophores, enables gaining information from structures of interest. Although the resolution in fluorescence microscopy is diffraction limited, some techniques were invented to overcome this resolution limit.

The following chapter describes the principles of fluorescence microscopy, starting with the processes that contribute to fluorescence with a focus on fluorophores and their physical and chemical properties. Subsequently different high-resolution techniques such as laser scanning microscopy (LSM) and stimulated emission depletion (STED) microscopy are presented.

2.1 Principles of Fluorescence

Fluorophores are small molecules with the ability to emit light. This usually happens as a consequence of excitation of those, usually aromatic molecules due to light exposure. There exists a great variety of different fluorophores, each of them absorbing light of a certain wavelength. The wavelength of the absorbed light is usually blue shifted with respect to the emission light wavelength. This property allows for utilizing fluorophores in microscopy.

To illustrate the different processes, which contribute to fluorescence, usually Jablonski diagrams are used (see Figure 1).
Figure 1: Jablonski diagram of different electronic and vibrational states. Arrows depict some of the transitions possible between these states. \( S_0 \) and \( S_1 \) refer to the singlet ground and excited state, respectively. \( T_1 \) depicts the first triplet state. The vertical lines illustrate the various transitions. Abbreviations: two-photon absorption (TPA), stimulated emission (SE), excited state absorption (ESA), inter-system crossing (ISC), triplet-triplet absorption (TTA). Revised from [8, 9].

The bold horizontal lines refer to the singlet ground (\( S_0 \)), first excited (\( S_1 \)) and triplet state (\( T_1 \)). The thin horizontal lines illustrate the fact that every electron in such an energy level can exist in different vibrational energy levels. The dashed lines within this image refer to non-radiative transitions. Absorption of light leads to excitation of electrons from ground to excited states (arrows pointing upwards). By contrast, light emission happens due to electrons going from excited to lower states (arrows pointing downwards). The reason for those arrows to be vertical is to show the instantaneous nature of emission and absorption processes, as those transitions occur within \( 10^{-15} \) s [9]. This is known as the Franck-Condon-Principle (illustrated in Figure 2), which states that the transitions most likely occur between states which are vertically above one another [10]. The reason therefore is that during electronic transitions, the cores will change its position only slightly.
Figure 2: Illustration of the Franck-Condon-Principle. Electron transitions occur most likely between states with (nearly) the same nuclear coordinate. This is due to the fact that the cores don’t change their position during electronic transitions, as they happen within $10^{-15}$ s. $v'$ and $v''$ denote the vibrational energy levels of the ground and excited state, respectively. $\Delta q$ is the distance between the core potential minima. Electrons are excited with a rate $k_{\text{exc}}$ while spontaneous emission happens with rate $k_{\text{em}}$. Undulated arrows represent vibrational relaxation.

2.1.1 Fluorophores

In fluorescence microscopy, the sample is the light source itself. Small molecules called fluorophores are the reason for this. Upon excitation with light from a laser, they emit light, which is detected and enables observation of i.e. living organisms in their natural environment.

Fluorophores can broadly be divided into two groups: intrinsic and extrinsic [9]. Contrary to extrinsic fluorophores, which are used for labeling of non-fluorescent material, naturally occurring dye molecules are called intrinsic. An example therefore is intrinsic protein fluorescence due to aromatic amino acids. In cases of either non-fluorescent samples or change of the optical properties from intrinsic samples, extrinsic fluorophores are used. Concerning proteins, it is often useful to label the sample using chromophores with other absorption and emission wavelengths than the amino acids. This allows to observe both, the labeled and unlabeled protein.
There exists a great variety of different fluorophores for covalent and non-covalent labeling of proteins.

Fluorophores have two important properties, namely quantum yield $\Phi$ and fluorescence lifetime $\tau$. The **quantum yield** is defined as the ratio of the number of photons emitted to the number of photons absorbed.

$$
\Phi = \frac{k_r}{k_r + k_{nr}} = \frac{k_r}{k_{fl}}
$$

(1)

In this formula, $k_r$ is the radiative rate of the fluorophore and $k_{nr}$ is the rate of all non-radiative decays. If the radiative rate is much greater than the rate of non-radiative decay, the quantum yield can be close to one [9]. The average time, a molecule spends in the excited state before returning to the ground state is called the **lifetime** of the excited state. It is an average value as fluorescence emission is a random process.

$$
\tau = \frac{1}{k_{fl}} = \frac{1}{k_r + k_{nr}}
$$

(2)

The natural lifetime of a fluorophore is defined as the lifetime of the fluorophore in absence of non-radiative decay.

In general it is important that the fluorophores provide sufficient photostability which is true for Alexa Fluor dyes that are used within the experiments for this diploma thesis.

There are several mechanisms that decrease the intensity of the fluorescence light, which are summarized under the term of **fluorescence quenching** [9]. This quenching can happen as a consequence of collisions of the fluorophore with other molecules (quenchers) within a solution. Static quenching, which is due to non-fluorescent complex formation of fluorophores and quenchers, and excitation light attenuation by fluorophores and other light absorbing molecules are other quenching processes [9].

Another process affecting the fluorophores ability to emit light is **photobleaching**. This is a dynamic process in which the fluorophores undergo photochemical modifications resulting in losing their ability to emit light. The bleaching rate depends on the excitation intensity [11].
2.1.2 Absorption

In fluorescence microscopy as well as in optical lithography, light i.e. photons are used to excite fluorescent molecules from the ground state to an excited state. This happens due to electron excitation from one molecular orbital to another. In polyatomic molecules there are several transitions of electrons. The participating molecular orbitals can be found in Figure 3.

There are three different ways for electrons getting excited. One is absorption due to non-binding electrons belonging to chromophore groups [10]. These chromophores are the part of a molecule which is responsible for its color as they absorb light of a certain wavelength [10]. Concerning C = C bonds, electrons are excited from a binding π – orbital to an anti-bonding π* – orbital which therefore is called π → π* – transition. This process requires energies corresponding to a wavelength of about 160 nm [12]. In the carbonyl group, an electron from the oxygen is excited from a non-bonding n – orbital to an anti-bonding orbital of the C = O bond [10, 12]. The absorption maximum is at about 280 nm [12].

Another possibility is absorption by binding electrons. The electrons are excited from a binding σ – orbital to an anti-binding σ* – orbital [8]. This usually requires high energies [10]. As the electrons are directly participating to the bond, σ → σ* - transitions can lead to dissociation of the molecule [10].
The third possibility is absorption by electrons in delocalized orbitals as it happens in benzol, where the electrons are delocalized over the whole molecule. Thereby, $\pi$–electrons are excited to a $\pi^*$–orbital due to light exposure ($\pi \rightarrow \pi^*$–transition) [10]. The absorption spectra of aromatic molecules shift to longer wavelengths with increasing size of the aromatic ring [10].

The mechanism of light absorption can either happen due to absorption of a single photon or multiphoton absorption (MPA). Absorption of more than one photon was first predicted as the special case of two-photon absorption (TPA) by Marie Göppert-Mayer in 1931 [13]. Photons can either be absorbed sequentially or simultaneously [14, 15]. In sequential absorption, the fluorophore is excited to a real intermediate state by the first photon, whereas in simultaneous absorption, this intermediate state is a virtual one [15]. Thus, TPA only happens, if there is a second photon absorbed during the lifetime of this virtual intermediate state. Therefore, a high photon density is required, which is realized using ultra-short pulsed lasers and high numerical aperture (NA) objectives to tightly focus the laser beam. Because of this requirements, TPA could not be verified until thirty years later [16], as in the 1960s lasers were invented [17].

### 2.1.3 Fluorescence Emission

As mentioned above, fluorophores are molecules with the ability to emit light after absorption of photons. This fluorescence emission light has some general characteristics, independent of the certain fluorophore.

One is the Stokes-shift [9], which appears as shift between the maxima of emission and absorption spectrum. Fluorescence emission has typically lower energies than the light used for excitation of the molecules. This is due to energy loss because of thermal relaxation of various vibrational excited states. Therefore the wavelength of the emitted light is longer (red shifted) with respect to the wavelength of the absorption light.

Another property of fluorescence emission (with only a few exceptions) is the fact, that the emission spectrum stays the same, independent of the excitation wavelength used. This is a consequence of Kasha’s Rule [18], which states that emission of photons happens most likely from the lowest excited state.
While comparing emission and absorption spectra, another common feature appears which is known as the mirror-image rule. It states, that the shape of the two curves is similar when mirrored [9, 10]. This is a consequence of relaxation to the ground state usually occurs to an excited vibrational ground state level. As spacing between vibrational energy levels is the same for ground and excited states, the shape is similar for absorption and emission spectra [9]. However, there are many exceptions of the mirror image rule.

### 2.2 Fluorescence Microscopy

Far field light microscopy, especially fluorescence microscopy, is an important and powerful tool, particularly in biology and life sciences. Thanks to fluorescence microscopy, investigation of biological samples such as living cells is possible. Therefore, fluorophores are used for labeling. They enable to distinguish the samples area of interest from the background by making use of different filters. The resolution achievable with fluorescence microscopy is limited to about 200 nm as a consequence of light diffraction. Due to the wave nature of light, point like objects will always appear not as point but blurred. This intensity distribution is called point spread function (PSF) [19, 20].

Most of the fluorescence microscopes used in life sciences are in epifluorescence configuration [21]. Excitation light is focused onto the sample (wide-field) by using an objective lens, which is as well collecting the emission light from above the sample. The reflected excitation light can be excluded by using appropriate filtering.

The low axial resolution is the main disadvantage of epifluorescence microscopes. As the whole sample gets illuminated, all fluorophores (from all layers) will emit at the same time. The signal originating from the focal plane is therefore overlaid by a high background.
2.2.1 Confocal Microscopy

![Principle of confocal microscopy](image)

**Figure 4: Principle of confocal microscopy.** The sample gets illuminated point like, which is achieved by pinholes (PH) in front of the excitation source and the detector. Scanning of the sample is performed either by sample or beam scanning.

In confocal microscopy, pinholes in front of the excitation source and the detector are used for increasing resolution and contrast. The principle is shown in Figure 4. By placing the pinholes in the focal plane of the objective lens, excluding of out-of-focus light can be achieved. As only emission light from the focal plane can be collected, confocal microscopy enables three-dimensional imaging with a lateral resolution enhancement of a factor of about 0.7 [22] and an axial resolution of one wavelength. Information is gained either by stage or beam scanning and a dichroic mirror is used to make sure that only light from the focal plane reaches the detector.

In comparison to epifluorescence microscopy the intensity of detected light is lower in confocal microscopy. Due to the point by point scanning, confocal microscopy can be time consuming. In both cases, photobleaching of the fluorophores is a limiting factor for resolution due to multiexposure.

2.2.2 Two-Photon Microscopy

In 1990, Denk et al. succeeded in overcoming the problems of confocal microscopy by combining two-photon excitation and laser scanning microscopy (LSM) [23]. In order to achieve two-photon absorption, the local intensity has to be very high, which means a high photon flux. This high intensity is provided by a femtosecond pulsed laser in combination with the tight focusing of the LSM. Fluorescence is generated only within the focal spot, as the intensity is high enough there [24].
Using two photons instead of one photon for the excitation of the fluorophores enables to achieve three-dimensional resolution comparable to confocal microscopy without intensity loss due to the pinhole [23]. Still, applying confocal pinholes can further increase resolution in three dimensions by a factor of ~0.7.

2.2.3 STED Microscopy

In 1994, Hell and Wichmann presented a method to overcome the diffraction barrier in far field optical microscopy [2]. They suggested deactivating the fluorophores in the outer rim of the focal spot by stimulated emission depletion (STED). Therefore, a second laser beam, the so called STED beam, is introduced. The wavelength of the STED beam is tuned to the fluorophores emission wavelength, which enables depletion of the excited state. This clearly has to be done before fluorescence emission occurs. The resulting PSF is called the effective PSF and is illustrated in Figure 5.

![Figure 5: Basic idea of stimulated emission depletion (STED) microscopy.](image)

Two laser beams are used for STED microscopy: the excitation beam with an ordinary PSF and the depletion beam shaped in a way, that the centre shows zero intensity. This leads to a narrowing of the PSF in two (donut shaped PSF) or three (“bottlebeam”) dimensions.

Abbreviations: stimulated emission depletion (STED), point spread function (PSF)

This technique was first experimentally carried out by Klar and Hell [25]. They were able to increase the lateral and axial resolution by a factor of 2 and 5, respectively, when compared to confocal microscopy. Recently a lateral resolution of 16 nm could be achieved [26]. The resolution achievable with STED microscopy can be derived by the following formula [26, 27]:

\[
\Delta r \approx 0.45 \frac{\lambda}{n \sin \alpha \sqrt{1 + I_{STED}/I_{sat}}}
\]

(3)
wherein $\lambda$ is the wavelength, $n$ the refractive index, $\alpha$ the lens semiaperture, $I_{\text{STED}}$ and $I_{\text{sat}}$ the depletion and saturation intensity, respectively. By this formula it can be seen, that the achievable resolution is theoretically unlimited.

Although the depletion efficiency is best when using a pulsed laser, this requires well temporarily synchronized excitation and STED beams. A more simple way for doing STED microscopy is to use a continuous wave (CW) laser as depletion beam source [28]. To achieve a resolution comparable to pulsed STED, the required intensity for CW-STED microscopy has to be four times higher. Thus the majority of photons is arriving at times where depletion already has taken place.
3 Optical Lithography

As optical lithography is often referred to as the reverse mechanism to microscopy, the principles needed to understand both fields are similar. This chapter provides an overview about optical lithography (i.e. radical polymerization). The materials used for lithography experiments will be presented. Also stimulated emission depletion (STED) lithography and other techniques to decrease structure size and resolution will be discussed.

3.1 Principles of Optical Lithography

Illumination of a material called photoresist allows for fabrication of polymer structures of desired size and shape. Generally, distinction of three different groups of photoresists can be made:

A **negative tone photoresist** is liquid before it gets illuminated. Exposure to light causes solidification of the photoresist due to a process called cross-linking of monomers. The unexposed regions remain liquid and can be removed by a subsequent development process [29].

On the contrary, **positive tone resists** liquefy when exposed to radiation. The unwanted resist is again removed in a development process [29].

**Dual tone resists**, as the name suggests, combine the properties of positive and negative resists. Initially solid, the resist stays unaffected by low excitation intensities, liquefies under medium exposure and gets solid again under high excitation intensities due to cross-linking processes [29–31].

3.1.1 Photoresist

A photoresist is usually composed of at least two materials, a monomer and a photo-initiator. When exposed to radiation, this combination of materials is changing its solubility [29].

The photoresists behavior can be described by a threshold model. If the exposure dose, which depends super-linearly on the intensity of incident light [8], reaches a certain threshold value, polymer structures will remain after the development process.
Reducing the exposure dose towards the threshold leads to a decreasing excitation volume and therefore smaller feature sizes. Consequently, the mechanical stability of the polymer structures will be reduced [32]. Figure 6 illustrates this behavior.

![Figure 6: Polymer conversion correlated with illumination cross section.](image)

The proximity effect is a second factor that influences the width of the fabricated structures. It states that the width is dependent on the resist and nearby objects. Therefore even non-exposed regions get a non-zero exposure dose [33]. In accordance to the above-mentioned properties of photoresists, two idealized cases can be distinguished [8]:

**Non-Forgetting Photoresist.** According to the law of reciprocity, the exposure dose, defined as \( E = \text{intensity} \times \text{exposure time} \), is directly proportional of the blackening of a photographic plate. This is fulfilled for remembering resists. As a consequence, \( E \) is summed up. In case of multiple exposures, the gelation process can be started even if the individual doses are below the threshold.

**Forgetting Photoresist.** There are deviations from the law of reciprocity, found by Schwarzschild [34]. He observed that when using very low intensities and long exposure times, photographic plates show no blackening (or at least lower blackening than expected). This behavior of a forgetting photoresist is called Schwarzschild effect.
3.1.2 Polymerization Process

Polymerization is a chemical reaction, which describes the formation of polymer chains or networks out of monomers. Monomers are the repeating unit within a polymer molecule and the degree of polymerization is the number of monomeric units per polymer molecule [35].

There are several monomers for polymerization available. These monomers have to be at least bifunctional and normally belong to one of the following groups [35]:

**Monomers with two (or more) functional end groups.** Examples for this group on monomers are amino acids (H₂O – Z – COOH), triamines (Y(NH₂)₃) or dichlorides (Cl–X–Cl). The letters X, Y and Z denote the bi-, tri- and tetrafuntional character of the substituents, respectively [35].

**Monomers with multiple bonds.** Those multiple bonds are mostly, but not exclusively, carbon-carbon double bonds. Examples for this group of monomers are propylene (CH₂=CHCH₃) or acetylene (HC≡CH).

**Cyclic monomers.** Those monomers are cyclic and have a heteroatom within the ring. Examples are tetrahydrofuran and ethylene oxide [35].

A common way to categorize polymerization processes is to distinguish between polymerizations with and without byproducts (denoted as L in Table 1) and the way polymer molecules (Pᵢ) react, respectively. During the growth step polymer molecules can either react only with monomers (M) or with other polymer molecules (Pⱼ) in addition.
Table 1: Overview of the categorization of different polymerization processes. $P_i$ and $P_j$ denote polymer molecules where $i$ and $j$ are the number of structural units, $M$ is designated a monomer and $L$ is a leaving molecule. Revised from [35].

<table>
<thead>
<tr>
<th></th>
<th>Step-growth polymerizations</th>
<th>Chain-growth polymerizations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polymerizations</strong></td>
<td>$P_i + P_j \rightarrow P_{i+j}$</td>
<td>$P_i + M \rightarrow P_{i+1}$</td>
</tr>
<tr>
<td>without byproducts</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IUPAC$^1$ name</strong></td>
<td>polyaddition</td>
<td>chain polymerization</td>
</tr>
<tr>
<td><strong>Polymerizations</strong></td>
<td>$P_i + P_j \rightarrow P_{i+j} + L$</td>
<td>$P_i + M \rightarrow P_{i+1} + L$</td>
</tr>
<tr>
<td>with byproducts created</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IUPAC name</strong></td>
<td>polycondensation</td>
<td>condensative chain polymerization</td>
</tr>
</tbody>
</table>

**Free Radical Polymerization**

The process of free radical polymerization can be characterized as chain-growth polymerization reaction.

Photoresists for free radical polymerization usually consist of a monomer and a photo-initiator. As the name suggests, the polymerization is initiated by free, namely unbound radicals, which exhibit an unpaired electron.

![Figure 7](image_url)

**Figure 7: Schematic representation of the free radical polymerization process.** In this scheme, $R$ denotes the photo-initiator and $R^*$ shows the free radical character of the initiator. $M$ stands for the monomer and as before the $*$ indicates a radical at the end of the monomer. $P$ denotes the polymer as final product of the polymerization process.

The process of radical polymerization can be described in three steps, schematically shown in Figure 7.

$^1$IUACP = International Union of Pure and Applied Chemistry

$^2$The term degree of polymerization refers to the number of monomeric units per polymer molecule.
First, **polymerization initiation** happens due to formation of free radicals. This can either be a self-initiation of the monomer (without any other initiator present) or a generation of free radicals due to thermal, photochemical or electrochemical reactions. The created radicals are able to break the double bonds of the monomer and link covalently in order to form chains [35]. To prevent self-initiation, which is present in for example methyl methacrylates, so called radical inhibitors can be used.

After initiation, the **propagation** of monomer chains linked by radicals starts. In radical polymerization this is done mainly in head-to-tail formation [35, 36].

This process continues until **termination** of the process occurs. There exist several possibilities for termination of polymerization, namely coupling of radicals and termination by the monomer. In the case of high concentrations of photo-initiators within the resist, coupling by initiator radicals has to be considered, too [35].

The chain length of the polymer (or degree of polymerization\(^2\)) can be controlled by the concentration of the photo-initiator within the resist. Using low concentrations of initiators will result in a high degree of polymerization as termination by coupling initiators can be neglected.

**Polymerization Kinetics**

In ideal kinetics, the polymerization rate is directly proportional to the concentration of monomers [35]. This is especially true for low monomer conversions. By increasing the monomer conversion in the range of 15 – 25% (e.g. in bulk materials with high viscosity) auto- (or self-) acceleration of the polymerization can be observed. Thus the polymerization rate (and degree of polymerization) is increased which can lead to a rise in temperature. This auto-acceleration is called **gel effect** (or Trommsdorff-Norrish effect) [35]. Increasing the monomer conversion even higher, the **glass effect** occurs. At this stage, the polymerizing system completely solidifies. Apart from reduction of radical diffusion and termination reactions, even the diffusion of monomers is reduced thus resulting in incomplete polymerization of monomers [35].

\(^2\)The term degree of polymerization refers to the number of monomeric units per polymer molecule.
3.1.3 Materials

Monomers. Table 2 shows some monomers available for free radical polymerization and the name of the resulting polymers.

<table>
<thead>
<tr>
<th>Structure of the monomer</th>
<th>Monomer name</th>
<th>Polymer name</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>methyl methacrylate</td>
<td>PMMA</td>
<td>Thermoplastics</td>
</tr>
<tr>
<td></td>
<td></td>
<td>poly(methyl methacrylate)</td>
<td></td>
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<tr>
<td></td>
<td>styrene</td>
<td>PS</td>
<td>Thermoplastics</td>
</tr>
<tr>
<td></td>
<td></td>
<td>poly(styrene)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vinyl acetate</td>
<td>PVA</td>
<td>Adhesives, coatings</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly(vinyl acetate)</td>
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<td></td>
<td>vinyl chloride</td>
<td>PVC</td>
<td>Thermoplastics</td>
</tr>
<tr>
<td></td>
<td></td>
<td>poly(vinyl chloride)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ethylene</td>
<td>PE</td>
<td>Fibers, thermoplastics</td>
</tr>
<tr>
<td></td>
<td></td>
<td>poly(ethylene)</td>
<td></td>
</tr>
</tbody>
</table>

There is a huge variety of monomers commercially available for free radical polymerization. Therefore, and because of the process being easy to control, free radical polymerization is the most important class of industrial polymerization.

Initiators. The photo-initiators used in polymerization processes are typically split in two groups: type I and type II initiators. The difference is the way radicals are formed. In type I initiators, two radicals are generated as a consequence of cleavage of a covalent bond (homolysis). In type II, hydrogen donors are used for the reaction with the triplet excited state of the initiator. Thereby radicals are generated [37, 38]. The initiation process is typically faster in type I than in type II initiators, as this process is bimolecular.
**Solvents.** Using solvents within the resist leads to dilution. Thus the monomer concentration gets lower which leads to a decrease of the polymerization rate. Additionally, chain transfer to polymer becomes less likely resulting in a lower degree of branching and a reduction of the gel effect [35]. Thus heat transport gets enhanced.

### 3.2 Two-Photon Lithography

In 1997, the process of two-photon lithography (2PP) was introduced for structuring with visible light [3] using negative photoresists. Since then lateral and axial feature size improved from 1.3 µm and 2.2 µm [3], respectively to feature sizes of 90 [39], 80 [40], [41] and 65 nm [42]. For structuring, pulsed lasers with different wavelengths are focused within the photoresist. The resist contains photo-initiators, which get excited by two photons. This leads to radical formation and the polymerization reaction gets started. Resolution achievable, which is defined by the minimum spacing between two structures (lines), is about 200 nm [43]. Utilizing 2PP also allows for three-dimensional structuring (Figure 8). Therefore, it has a large variety of application areas including photonic crystals, optical data storage and fabrication of tips for atomic force microscopy, to name just a few [44].

![Figure 8: SEM image of a three dimensional structure fabricated by using 2PP.](image_url)

The photoresist for structuring is a 80/20 mixture of PEG-DA (Polyethylene (glycol) diacrylate, Sigma Aldrich Co.) and PETTA (pentaerythritol tetraacrylate, Sigma Aldrich Co.) including 3 wt% IC (Irgacure 819, BASF, Ludwigshafen, Germany) as photo-initiator for 2PP. The excitation power used is $P_{ex} = 6$ mW (@ 780 nm). Scale bar: 10 µm
3.3 STED and STED inspired Lithography Techniques

In order to push both, resolution and feature size in optical lithography, to new limits, the idea of STED microscopy was adapted to lithography. This approach and STED inspired techniques are pursued by several groups [4, 32, 45–47].

3.3.1 STED Lithography

The principle of STED lithography is shown in Figure 9. The photo-initiator is excited from $S_0$ to an excited state $S_1^*$ by two photon (or multiphoton) absorption. After relaxation from $S_1^*$ to $S_1$ this leads to inter system crossing (ISC) to a long-lived, reactive triplet state $T_1$. As a consequence, radicals are generated which start the polymerization process. This cross-linking of monomers is an irreversible process.

![Figure 9: Principle of STED lithography](image)

The basic idea of STED is now to prevent ISC and therefore radical generation and polymerization. Thus the initiators are forced from $S_1$ to $S_0$ by stimulated emission before ISC occurs. This is possible when using sufficient photo-initiators.

Today, the only initiator found capable of true STED lithography is 7-diethylamino-3-thenoyl coumarin (DETC) [48] which is also a fluorophore. Surprisingly, this initiator shows an effective three-photon excitation behavior at high repetition rates and an effective four-photon behavior at low repetition rates [49]. Fluorophores showing a two-photon behavior can be depleted [8], whereas multiphoton polymerization may lead to direct radical generation that cannot be depleted.
For efficient stimulated emission, high depletion laser intensities with temporarily aligned pulses or CW-lasers are used. In order to restrict the excitation volume in two or three dimensions phase masks are used. Figure 10a shows the phase mask used for shaping the STED beam into a donut, which allows for reducing the feature size in two dimensions. The circularly polarized incoming light is partly delayed (depending on the azimuthal angle). The central region of the beam has zero intensity. Using so called ‘bottlebeam’ phase masks shown in Figure 10b enables structure size reduction in three dimensions. The inner part of the incoming light is phase delayed. Destructive interference in the focal region leads to zero intensity in the middle.

![Phase masks used for shaping the depletion beam.](image)

**Figure 10: Phase masks used for shaping the depletion beam.** a shows the $2\pi$ spiral phase mask used for reducing feature size in two dimensions. This is possible due to shaping of the STED beam into a donut (zero intensity in the center of the beam). b depicts the annular phase mask for feature size reduction in three dimensions. Revised from [50].

Using STED lithography enables to fabricate structures with sizes of 55 nm (lateral) [4] and 53 nm (axial) [50]. The lateral resolution achieved is 120 nm [4].

### 3.3.2 RAPID Lithography

Another possibility to achieve high resolution in lithography is called resolution augmentation through photo-induced deactivation (RAPID) lithography [45]. The principle is shown in Figure 11a. This technique is not a true STED as it gets along with light of a single wavelength but two different pulse durations. First, the photo-initiator gets excited with a femtosecond laser pulse. A second, stretched pulse with pulse duration of >50 ps is used for prevention of efficient two photon absorption (TPA). Due to lone photon absorption by a long lived intermediate state, the intermediate state is hindered from producing cross-linked polymers. This process allows utilizing a CW-laser for depletion. The need for temporarily aligned pulses is therefore eliminated.
Figure 11: Principle of STED inspired lithography techniques [8]. a In RAPID (resolution augmentation through photo-induced deactivation) lithography the photo-initiator gets excited using two photon absorption (TPA). This leads to generation of radicals in a long lived intermediate state which is deactivated using light excitation. Thus it prevents cross linking of polymers. b 2PII (two-color photo-initiation / inhibition) lithography uses one photon absorption (OPA) of one wavelength to excite the initiator and another wavelength for excitation of the inhibitor molecules. First one leads to radical formation capable of starting the polymerization process. The radical generated from the inhibitor molecules are terminating the cross linking process.

3.3.3 2PII Lithography

Two-Color photo-initiation / inhibition lithography (2PII) is another STED inspired technique presented by Scott et al [32]. The principle is shown in Figure 11b. A photo-initiator is excited using one photon absorption (OPA) for creation of radicals (R*). In comparison to the techniques introduced in the above sections, a photo-inhibitor is used in addition. Upon excitation of the inhibitor with a second wavelength, reactive radicals (Q*) were generated, which terminate the polymerization process.
4 Methods and Materials

This chapter first describes the experimental setups used to fabricate and characterize the polymer structures. In the following, the materials used within the experiments are described. The last part of this chapter gives a brief overview about the different structures fabricated.

4.1 Experimental Setups

All multiphoton and STED lithography experiments and parts of the atomic force microscopy (AFM) measurements described within this chapter were performed at the Institute of Applied Physics, JKU Linz. The fluorescence microscopy measurements and parts of the AFM measurements were taken in collaboration with Birgit Plochberger, Institute of Applied Physics, Vienna University of Technology and were also performed in Vienna.

4.1.1 Lithography Setup

A schematic image of the STED lithography setup is shown in Figure 12. The excitation light used for multiphoton polymerization is generated by an ultra-short pulsed fiber laser with a wavelength of 780 nm (FemtoRay780, 50 MHz repetition rate, 100 fs pulse duration, Menlo Systems GmbH, Munich, Germany). The optical output power is 39 mW. Power adjustment of the excitation beam is provided by an acousto-optic modulator (AOM; Q1133, Isomet, Springfield, VA., USA). The depletion laser is a continuous wave (CW) diode pumped Nd:YVO₄ laser that emits light at a wavelength of 532 nm (Verdi-V5, Coherent, Santa Clara, CA, USA). The maximum output power is 5 W. A combination of two stages is used to perform sample translation. A three axes piezo stage (P-562.3CD, PhysikInstrumente PI, Karlsruhe, Germany) with a repeatability of 2 / 2 / 4 nm (in x / y / z) and a travel range of 200 x 200 x 200 µm, is holding the sample. This stage is mounted on top of a motorized stage (M-686.D64, PhysikInstrumente PI, Karlsruhe, Germany) with a travel range of 25 x 25 mm. The stages are driven in closed loop with two controllers (E710.3CD and C-867.260, both from PhysikInstrumente PI, Karlsruhe, Germany).
Figure 12: Schematic image of the STED lithography setup. Two lasers are used, a continuous wave laser with 532 nm wavelength and a pulsed laser with 780 nm and 100 fs pulse duration. Pinholes within the optical path of each laser enables for mode-filtering. Phase plates allow shaping of the depletion beam in a way that there is zero intensity in the center (‘donut’).

Abbreviations: continuous wave (CW), acousto-optic modulator (AOM), pinhole (PH), $2\pi$ phase plate (PP), avalanche photo diode (APD) with 62.5 µm glass fiber, personal computer (PC).

**Beam Shaping.** Using a $2\pi$ spiral phase mask (RPC Photonics, Rochester, NY, USA) enables to shape the point spread function (PSF) of the depletion beam in such a way, that the center of the beam has ideally zero intensity. Therefore the depletion beam needs to be circularly polarized, which is realized using a $\lambda/4$ plate (Thorlabs-Inc., Newton, New Jersey, USA).

**Objective Lens.** An oil immersion objective lens (Zeiss α-plan Apochromat, 100x, numerical aperture NA = 1.46 oil immersion lens) is used with immersion oil (Immersol 518 F, refractive index $n = 1.518$). The objective lens is aberration corrected for cover slips (Menzel Gläser, Braunschweig, Germany) with a thickness of 170 µm.

**Detection.** For detection, a glass fiber (62.5 µm diameter) is connected to an avalanche photodiode (APD) (APD-SPCM-AQRH, PerkinElmer Optoelectronic Inc., Waltham, Massachusetts, USA). The fluorescence signal from the sample is focused onto that glass fiber. Readout is taken on a multifunctional data acquisition (DAQ) module (NI USB-6229 BNC, NI National Instruments, Austin, Texas, USA). This DAQ module is also used for positioning- and shutter-control as well as for control of the AOM.
Software. For sample positioning, recording of images and controlling the writing process, a custom built LabView (LabView 2011, National Instruments Corporation, Austin, Texas, USA) program is used (originally written by Richard Wollhofen, Institute of Applied Physics, JKU Linz).

4.1.2 Confocal Laser Scanning Microscopy

Using confocal laser scanning microscopy (LSM 700, Carl Zeiss AG, Oberkochen, Germany) enables to indirectly show the functionalization of the fabricated polymer structures. Therefore, the prepared structures were labeled with mercapto reactive Alexa647. Images were taken using a 64x objective NA = 1.4 Oil DIC Plan-Apochromat. The samples were illuminated with lasers of 488 nm and 647 nm wavelength (Laser modules LSM 700). After appropriate filtering, the emitted signals were imaged on photomultiplier tubes (PMTs, Spectral Detection LSM 700).

Operation of the LSM was done by using Zen data processing software (Carl Zeiss AG, Oberkochen, Germany). The images are background corrected and detected signals were averaged.

4.1.3 Combined Fluorescence Microscopy and Atomic Force Microscopy

Before measuring, the sample was sealed by a home built chamber and rinsed with phosphate-buffered saline (PBS) with pH 7.4. To ensure appropriate tip positioning, fluorescence microscopy was used right before the AFM measurements.

Measurements were taken on an AFM system (Nanowizard 3, JPK Instruments AG, Berlin, Germany) mounted on an Axiovert 200 inverted microscope (Carl Zeiss AG, Oberkochen, Germany). Two objectives were used with the microscope: a 100x NA = 1.45 oil-immersion Plan-Apochromat TIRFM (Olympus, Tokyo, Japan) and a 20x NA = 0.8 Plan-Apochromat objective (Carl Zeiss AG, Oberkochen, Germany).
The samples were illuminated in epifluorescence configuration with light of 488 nm and 647 nm wavelength both with 250 mW from a diode laser (Toptica Photonics, Munich, Germany) or light of 532 nm wavelength from a solid state laser ( Millennia X, Spectra Physics, Mountain View, CA) with intensities of 3-10 kW/cm². For the 532 nm light an AOM (1205C, Isomet, Springfield, VA, USA) is used for control of illumination times between 1 and 5 ms. Uncoated silicon cantilevers (MSNL-10, Bruker Corporation, Billerica, USA). JPK data software was used for image processing. Height images were line-fitted as required.

4.1.4 Atomic Force Microscopy

Height profiles of the depletion patterns were performed using atomic force microscopy (AFM) (Nanowizard 3, JPK Instruments AG, Berlin, Germany).

AFM measurements and data evaluation was performed using the JPK software.

4.1.5 Scanning Electron Microscopy

The STED lithographically fabricated polymer structures were analyzed using a scanning electron microscope (SEM) (Zeiss Smart-SEM:Supra 55VP, Carl Zeiss AG, Oberkochen, Germany). The polymer samples have to be coated with conductive materials. We used a platinum coating that is achieved by sputtering of platinum targets in argon plasma (Emscope Laboratories Ltd, Ashford, Kent, England), typically two minutes at 20 mA. The thickness of the metal coating is about 10 nm.

4.2 Materials

The photoresists for the experiments are basically composed of a monomer and the photo-initiator. As an additional component, different concentrations of bifunctional clusters (methacrylates and mercapto groups in varying proportions as ligands) were added.
4.2.1 Photoresist

**Monomer.** The material used as monomer in the photoresist is pentaerythritol triacrylate (PETA) (Sigma-Aldrich Co., St. Louis, USA). As the name suggests, PETA consists of pentaerythritol with three acrylate groups as ligands. These groups form polymer networks in the polymerization process by reactive π-bonds. The chemical structure of the monomer is shown in Figure 13.

PETA appears as a colorless fluid and is delivered with 300 – 400 ppm of monomethyl ether hydroquinone (MEHQ). MEHQ prevents premature polymerization as benzoquinone is created due to oxidation by oxygen. Benzoquinone itself is an inhibitor, which hinders the polymerization initiation due to delivering radicals which are unable to start the polymerization process [35].

![Chemical structure of the monomer and inhibitor](image)

**Figure 13: Chemical structure of the monomer and inhibitor used within the photoresist.** a shows the monomer pentaerythritol triacrylate (PETA). PETA has three acrylate groups as ligands and forms polymer networks in the polymerization process. b shows the inhibitor monomethyl ether hydroquinone (MEHQ).

**Photo-initiator.** The initiator for the radical polymerization process used is 7-diethylamino-3-thenoylcoumarin (DETC) (Acros Organics, Geel, Belgium). The chemical structure of DETC as well as the emission and absorption spectra of DETC in PETA are shown in Figure 14. DETC belongs to the group of keto-coumarins. The additional ketone group is common in photo-initiator molecules as it promotes the polymerization initiation process.

**Photoresist.** PETA is mixed with 0.25 wt% DETC, which appears as yellow powder. In order to dissolve the initiator within the monomer, both of them are mixed and heated up to a maximum temperature of 95°C. Additionally stirring for about 25 minutes is applied. A treatment with ultrasonic waves can be used as well for dissolving the initiator within the monomer.
4.2.2 Metal Oxo Clusters

The clusters used within the experiments were synthesized, analyzed and provided by Johannes Kreutzer, Institute of Material Chemistry, Vienna University of Technology.

Zirconium Clusters. The structure of the Zr₄O₂(OMc)₁₂ cluster (Zr4, OMC = methacrylate) is shown in Figure 15.

![Figure 14: Photo-initiator 7-diethylamino-3-thenoylcoumarin (DETC). a Chemical structure of DETC. b Emission (black) and absorption (orange) spectrum of PETA with DETC. The excitation beam in the lithography experiments has a wavelength of 780 nm and the depletion beam with a wavelength of 532 nm. Revised from [46].](image)

![Figure 15: Image of the zirconium cluster and chemical structure of its ligands [51]. a Zr₄O₂(OMc)₁₂ cluster (Zr4). Hydrogen atoms are omitted for clarity. b Chemical structure of the ligands available for this cluster. The upper image shows the methacrylate ligand which is used within the polymerization process for fabrication of cluster crosslinked polymers. The lower image shows the 3-mercaptopropionic acid which is used for functionalization of the cluster with thiol groups (SH).](image)
The cluster consists of four zirconium atoms and it has twelve methacrylate ligands, which are used within the polymerization process for fabricating cluster crosslinked polymers. The cluster appears as slightly yellow powder and has a molecular mass of 2305.2 g mol\(^{-1}\).

Within the photoresist, those clusters serve as crosslinkers because of the high density of polymerizable methacrylate ligands. The clusters are embedded into the polymer, without loss of their structural integrity [52, 53].

**Cluster Preparation.** All preparations regarding clusters and ligand exchange were performed in nitrogen atmosphere.

\(\text{Zr}_4\) was prepared as reported in [54]. The reaction mixture was stirred for one hour at ambient temperature. The product crystallizes within twenty four hours with a yield of 87% of \(\text{Zr}_4\). The cluster was dried under vacuum and dissolved in a small amount of \(\text{CH}_2\text{Cl}_2\) until a clear solution was obtained and precipitated from hexane. Free acid was removed by washing repeatedly with toluene.

**Ligand exchange.** The methacrylate ligands can partially be exchanged by mercapto groups. These exchange reactions were performed according to [55]. Synthesis of clusters with different concentrations of 3-mercaptopropionic acid leads to clusters with different amounts of thiol groups.

Three different thiol functionalized \(\text{Zr}_4\) clusters are compared with respect to their depletion efficiency:

- **ZrSH1**: 13% thiol groups as ligands: \(\text{Zr}_4\text{O}_2(\text{OMc})_{10}(\text{OPrSH})_2\) has on average two mercapto groups and ten remaining methacrylate groups as ligands.
- **ZrSH2**: 24% thiol groups as ligands: \(\text{Zr}_4\text{O}_2(\text{OMc})_9(\text{OPrSH})_3\) has on average three mercapto groups and nine methacrylates as ligands.
- **ZrSH3**: 65% thiol groups as ligands: \(\text{Zr}_4\text{O}_2(\text{OMc})_3(\text{OPrSH})_9\) has on average three SH groups and nine remaining methacrylate groups as ligands.

Exact determination of ligands is possible by destroying the cluster core after the exchange reaction. The integrals of the free acid then are compared.

The second cluster used within the experiments is a titan-zirconium cluster (\(\text{TiZr}\)). A more detailed description of this cluster can be found in the appendix.
4.2.3 Solvent

Dissolving of clusters was performed using anhydrous dichloromethane (or methylene chloride, DCM) with max. 0.001% H₂O (VWR International LLC, Radnor, Pennsylvania, USA). The use of anhydrous solvents is necessary, as the clusters start to decompose in contact with water and humidity. This is particularly true for clusters with a high number of thiol groups as ligands (i.e. ZrSH₃ and TiZr clusters with 87% thiols).

After dissolving the clusters in methylene chloride (100 µl DCM per 5 mg cluster) the photoresist (PETA with 0.25 wt% DETC) was added. For the experiments, cluster ratios of 3 wt% and 1 wt% were prepared, respectively. In order to get rid of the methylene chloride within the photoresist, 10 µl of the resist were pipetted onto a glass slide and then put in vacuum (about 100 mbar) for about 20 minutes.

4.2.4 Alexa647-Maleimide

In order to show the thiol functionalization of the polymerized structures thiol reactive Alexa647-maleimide linkers were used. Alexa Fluor 647 has a peak excitation wavelength of 650 nm and a peak emission wavelength of 665 nm [56]. The maleimide group binds covalently to accessible thiols within the polymer structures. Subsequently, the fluorescence signal of each structure was measured using confocal LSM. Figure 16 shows a sketch of the labeling process.
Figure 16: Sketch of the labeling process of the lithographically structured polymers with thiol reactive Alexa647. The photoresist used for structuring was either composed of PETA/DETC doped with 1 wt% ZrSH2 (27% of the methacrylate groups exchanged against 3-mercaptopropionic ligands) or 1 wt% Zr4. PETA with 0.25 wt% DETC is used as a negative probe. The structures were incubated for 30 minutes in PBS solution (pH 7.4) containing 5 mM Alexa647-maleimide. The maleimide group binds covalently to accessible thiols within the polymer structure.

4.3 Sample Preparation

Given excitation and depletion powers were measured before entering the objective lens. As a photoresist, PETA with 0.25 wt% DETC and 300-400 ppm MEHQ was chosen, because of the ability to use it in STED lithography experiments [6, 7, 57]. This ‘basic’ photoresist is doped with different concentrations of metal oxo clusters within the experiments for this diploma thesis.

4.3.1 Feature Size

For determining the minimal structure size of cluster crosslinked polymers, the beam of the depletion laser is shaped into a donut by using a 2\pi spiral phase mask. Using both lasers, lines with different excitation and depletion powers were written. To examine the difference in line thickness, the depletion laser was manually chopped during the writing process.

The so fabricated structures were characterized using the SEM. Therefore, the samples were coated with platinum of 10 nm thickness.
4.3.2 Depletion Efficiency

In order to compare the different photoresists, so called depletion test patterns were fabricated. Figure 17a shows an example for such test structures. Two lasers (780 nm and 532 nm) with ordinary PSFs were used for writing. Simulations of the PSFs used can be found in Figure 17b. The horizontal axis of the depletion patterns represents the excitation powers, while the vertical axis shows different depletion powers $P_{\text{STED}}$, used for structuring. In the experiments, excitation powers from 3.8 mW to 4.2 mW in steps of 0.1 mW were used. Depletion powers were increased from 0 mW (which can be compared to the first, bottom line in Figure 17a) to a maximum of 27 mW (within the experiments) in 42 similar steps. This leads to a region of fully depleted lines in the middle of the depletion patterns. Further increasing of $P_{\text{STED}}$ finally leads to residual absorption of the green laser which manifests in polymerization enhancement and therefore increased structure size.

![Image of a depletion test pattern including height profile from the lines of each column and PSFs used for writing](image)

**Figure 17:** SEM and AFM (inset) image of a depletion test pattern including height profile from the lines of each column and PSFs used for writing. 

* a Every column is fabricated using a constant excitation power (@ 780 nm) and each line is created with different, increasing depletion powers (@ 532 nm). Two ordinary PSFs are used, thus reducing the line height while increasing the depletion powers. This works until full depletion of lines occur. When increasing the power of the depletion beam above a certain value, most likely absorption of the green laser occurs which manifests in increasing line heights. Scale bar: 10 µm. 

* b Image of simulated PSFs in x-y-direction. For fabrication of the depletion test patterns, two ordinary PSFs were used. This allows for complete polymerization suppression in a certain region of the test patterns. Scale bar: 300 nm
From measuring the height of the lines from each column so called depletion curves were obtained. This enables to compare the different photoresists and characterize the effect of cluster doping on the depletion efficiency of PETA/DETC.

The feature height was measured with the AFM (Nanowizard 3, JPK Instruments AG, Berlin, Germany).

### 4.3.3 Thiol Functionalization

In cooperation with Birgit Plochberger, Institute of Applied Physics, Vienna University of Technology, confocal LSM and combined fluorescence and AFM measurements were carried out in order to show the thiol functionalization of the fabricated polymer structures.

For the experiment, the dye stock solution was dissolved with distilled water to obtain a final concentration of 10 mM Alexa647-maleimide. Then the thiol functionalized structures and the negative probe (structures fabricated with PETA and 0.25 wt% DETC) were incubated for 20 minutes and subsequently rinsed with water. Hereafter, confocal LSM measurements, with laser wavelengths of 647 nm and 488 nm, respectively, were performed.

Woodpile structures with 20 µm side length and four layers (1 µm height each layer) were created using two-photon polymerization (excitation power 4 mW @ 780 nm). The woodpile structures were chosen in order to minimize the background signal due to unspecific binding of the fluorescent linkers to the glass surface.

Two dimensional lines were chosen to investigate the thiol functionalization of STED lithographically written structures. A part of the lines was done by using both lasers. To point out the difference in line thickness due to STED, the depletion beam was donut shaped by using the 2π spiral phase mask. For structuring excitation powers of 3.2 mW and depletion powers of 7.8 mW were used.

Images of the woodpiles have been taken on LSM 700 and all images of the lines were obtained by using a custom made single molecule fluorescence microscopy setup.
5 Results

In this section, the results of the lithography experiments are shown. The main goal of these experiments is the direct functionalization of polymer structures with mercapto groups via embedding of zirconium and titan-zirconium clusters within the photoresist, respectively. The clusters carry different functionalities: methacrylates and mercapto groups, which are present in different concentrations. Using cluster doped photoresists allows for STED lithographically fabricated structures with minimum feature sizes of about 70 nm.

To investigate the dependence of feature size as well as depletion efficiency and STED ability from cluster concentration (clusters with varying concentrations of mercapto groups), different photoresist were used for structuring of depletion test patterns. Those patterns are arrays of lines, fabricated with different excitation and depletion powers.

Given excitation $P_{\text{ex}}$ and depletion $P_{\text{STED}}$ powers were measured in front of the objective lens.

5.1 Feature Size

A maximum of 5 wt% of clusters (TiZr or Zr4) can be dissolved within the photoresist. Anyway, with this cluster concentration, only MPP can be used to fabricate cluster-crosslinked polymer structures. In order to use STED lithography for structuring, lower cluster concentrations have to be used. The concentration limits can be obtained from Table 3.

<table>
<thead>
<tr>
<th>Cluster Concentration</th>
<th>Zr4 Cluster</th>
<th>Ti4Zr4 Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 wt%</td>
<td>MPP</td>
<td>MPP</td>
</tr>
<tr>
<td>4 wt%</td>
<td>MPP and STED</td>
<td>MPP</td>
</tr>
<tr>
<td>2.5 wt%</td>
<td>MPP and STED</td>
<td>MPP and STED</td>
</tr>
</tbody>
</table>
Lines fabricated using STED lithography show a maximum narrowing from 100 nm to 72 nm, which represents a 28% change in line thickness (see Figure 18). The excitation power $P_{\text{ex}}$ used is 3.2 mW and the depletion power $P_{\text{STED}}$ is 7.8 mW. The photoresist used for fabricating those lines is PETA with 0.25 wt% DETC doped with 2.5 wt% ZrSH2 (24% thiol groups as ligands). Data evaluation was taken out using scanning electron microscopy (SEM).

![Figure 18](image)

**Figure 18:** TPP and STED lithographically fabricated lines. To achieve a narrowing of lines, a phase mask was introduced within the optical path of the depletion laser. In order to examine the change in structure size, the green laser was interrupted repeatedly during the writing process. The red and green lines in the image refer to the excitation (@ 780 nm) and depletion (@ 532 nm) beam, respectively. Minimum feature size of 72 nm and a line thickness change of 28% can be obtained from these experiments. Scale bar: 200 nm

Further decreasing of the excitation power results in decreasing feature sizes at the expense of mechanical stability of the structures. The line shown in Figure 19 was fabricated using $P_{\text{ex}} = 2.5$ mW and $P_{\text{STED}} = 7$ mW with partially blocked depletion beam. The line width obtained by SEM is about 66 nm.

![Figure 19](image)

**Figure 19:** TPP and STED lithographically fabricated two dimensional structures. The line was fabricated with an excitation power of $P_{\text{ex}} = 2.5$ mW and $P_{\text{STED}} = 7$ mW. The minimum line width which could be estimated is 66 nm.
5.2 Depletion

In order to compare the photoresists doped with different clusters, depletion test patterns were fabricated. Two ordinary PSFs were used for the depletion experiments. This leads to a STED induced shrinkage of the polymer volume as can be seen in Figure 17. Each column of this test pattern refers to excitation powers of 3.8 to 4.2 mW (@ 780 nm) and each line has different, increasing depletion powers (@532 nm). Figure 20 shows the depletion curves for photoresists doped with 1 wt% ZrSH1 and 3 wt% ZrSH2, respectively. The depletion curves are plots of the feature height versus the depletion power and are obtained from the depletion test patterns by AFM (compare Figure 17). Data evaluation allows for characterization and comparison of the different photoresists.

Figure 20: Resulting depletion curves for photoresists doped with 1 wt% ZrSH1 and 3 wt% ZrSH2. For structuring, excitation powers of 3.8 to 4.2 mW (in steps of 0.1 mW) were used. The depletion powers are increased from 0 to about 23 mW (for ZrSH1) and 17 mW (for ZrSH2). a Full depletion of lines occurs for 3.9 mW excitation power and below. The depletion power needed is in a range of about 2 to 13 mW. The line height increases when increasing the depletion power most likely because of residual absorption of the depletion beam. b Doping of the photoresist with 3 wt% ZrSH2 hinders full depletion in the observed excitation power range.
Figure 20a and 20b show depletion curves for structures written with photoresists doped with 1 wt% ZrSH1 and 3 wt% ZrSH2, respectively. Different curves refer to different excitation powers (from 3.8 to 4.2 mW). While increasing the depletion power, the line height decreases until full depletion of lines occurs. Increasing $P_{\text{STED}}$ leads to an enhancement of the polymerization reaction, most likely due to residual absorption of the green laser (@ 532 nm).

It should be noted that full depletion of lines is possible for 1 wt% ZrSH1 with excitation powers below 4 mW whereas structures doped with 3 wt% ZrSH2 show no full depletion at all in the observed excitation power range from 3.8 to 4.2 mW. The data indicates that higher cluster / mercapto concentration increases the residual absorption of the depletion beam and reduces the region of fully depleted lines.

When comparing the same concentration (1 wt% of clusters) within the photoresist, clusters with a higher concentration of mercapto groups (ZrSH2) show lower depletion efficiency, as can be seen in Figure 21.

![Figure 21: Comparison of depletion curves fabricated with 4.1 mW excitation power with different photoresists. The green and blue curves refer to photoresists doped with 1 wt% and 3 wt% of clusters (13% and 24% of thiol groups). Data indicates that the higher thiol concentration within the photoresist leads to lower depletion efficiency. Comparison of different concentrations of the same cluster within the photoresist (green and pink curve) shows a similar tendency. Using a higher amount of clusters leads to increased structure sizes.](image-url)
5.3 Functionalization of Polymer Structures

To prove whether the mercapto groups are still accessible after the polymerization process, confocal laser scanning microscopy (LSM) measurements are carried out. Therefore the mercapto groups of the fabricated structures have been labeled by fluorescein-functionalized maleimide for 20 minutes. The fluorophore used here is Alexa Fluor® 647.

Figure 22: Confocal LSM images of woodpile structures fabricated with different photoresists. The woodpile structures have a side length of 20 µm and a total height of 4 µm. Using confocal LSM enables to exclude the background and image single layers of the woodpile structure. a Confocal images and intensity analysis of woodpiles written with PETA and 0.25 wt% DETC and PETA/DETC doped with 1 wt% Zr4 and 1 wt% ZrSH2, respectively. Specific binding of Alexa647-maleimide and the woodpile structure was quantified by a confocal LSM700. The LSM fluorescence image shows the attachment of the Alexa647-maleimide functional group. For comparison of structure size under the assumption that feature size correlates with the intensity, images were taken @ 488 nm (DETC fluorescence) and @ 647 nm. Scale bar: 5 µm. b The bar diagram shows the background corrected fluorescence signal of the three structures. The Alexa647 signal is depicted in dark grey, the DETC signal in grey.
Three different samples were used in order to show the accessibility of thiol groups after the polymerization process: woodpile structures fabricated via two-photon polymerization (2PP) of the photoresist doped with 1 wt% of $\text{Zr}_4$ clusters (without thiols), structures with 1 wt% of $\text{ZrSH}_2$ clusters (with mercapto groups) and structures fabricated with PETA and 0.25 wt% DETC as a negative probe. Confocal LSM images of the woodpile structures tested can be found in Figure 22a. The woodpiles were structured with 4 mW (@ 780 nm) excitation. For development of the structures, the glass slide was rinsed with methylene chloride. Excitation was taken out with wavelengths of 647 nm and 488 nm, respectively. The bar diagram in Figure 22b shows the background corrected fluorescence signal of the three structures. The excitation at 647 nm shows a thirtyfold increased fluorescence signal for the mercapto functionalized structures compared to the unreactive structures.

In a second experiment, two-dimensional structures (lines) were fabricated via multiphoton and STED lithography. Therefore, the $\text{ZrSH}_1$ doped photoresist was used. The control structures were fabricated with PETA and 0.25 wt% DETC.

![AFM and fluorescence images of STED lithographically fabricated lines.](image)

*Figure 23: AFM and fluorescence images of STED lithographically fabricated lines.*

*a* Image of two dimensional lines written with PETA with 0.25 wt% DETC. These structures are used as negative probe. The lines in the fluorescence image (inset) appear dark as DETC does not exhibit any fluorescence @ 647 nm. *b* Image of structures fabricated with a cluster doped photoresist (1 wt% $\text{ZrSH}_1$). It can be seen, that the line intensity from the fluorescence signal due to Alexa647-maleimide functionalization corresponds to the lines scanned with the AFM. Scale bars: 1 µm.
Figure 23 shows fluorescence and atomic force microscopy images of the lines. The line intensity determined from the Alexa647-maleimide functionalization corresponds to the AFM scanned line width. The control structure shows no fluorescence. However, the background which is caused by unspecific binding of the fluorophores to the glass surface hinders an exact quantification of the signals.

5.4 Metallization of cluster doped polymer structures

One reason for functionalizing polymer structures is to subsequently coat the structures with metal. Because of the mercapto-functionalized structures, gold was chosen for metallization.

The structures for those experiments are fabricated using PETA with 0.25 wt% DETC doped with 1 wt% and 5 wt% TiZrSH1 clusters, respectively. Figure 24 shows a schematic image of the gold nanoparticle (NP) synthesis and the metallization of two-dimensional functionalized structures.

We tried to realize a gold coating using 10 mM gold (III) chloride trihydrate (H\text{AuCl}_3•3\text{H}_2\text{O}, Sigma-Aldrich Co., St. Louis, USA) dissolved in ethanol. Subsequently, different reducing agents were added. Three different reduction processes were tested within the experiments (procedures loosely inspired by [58]):

- Reduction using UV light
- Reduction using a mild reducing agent: slow reduction using ascorbic acid (Sigma Aldrich Co.)
- Reduction with strong reducing agent: fast reduction using sodium borohydride (NaBH\text{4}, Sigma Aldrich Co.)

The thiol functionalized structures then were put overnight in the various solutions. Evaluation was carried out using dark field microscopy. The results of the experiments are shown in Figure 25.
Figure 24: Schematic image of the synthesis of gold nanoparticles (NP) and metallization of two dimensional thiol functionalized polymer structures. The gold (III) chloride trihydrate is reduced by use of a reducing agent. In this experiment, three different agents are used: UV light, ascorbic acid and NaBH₄. The polymers were fabricated by using photoresists with 1 wt% and 5 wt% of TiZrSH₁ clusters, respectively. The samples are put overnight in the solutions with different reducing agents.

Figure 25: Results of the experiments on metallization via gold (III) chloride trihydrate using different reducing agents. The photoresist used for structuring is PETA with 0.25 wt% DET C doped with 1 wt% and 5 wt% TiZr clusters (with thiol groups providing functionalization). Dark field microscopy was used for imaging. Scale bar: 5 µm.
Usage of the strong reducing agent NaBH₄ results in a fast formation of gold clusters rather than a coating of the functionalized structures. The reduction process most likely happens too fast. Therefore we exclude the NaBH₄ in following experiments regarding metallization of functionalized polymers.

UV radiation as reducing agent also seems not to lead to the desired metal coated functional structures. The structures fabricated with 5 wt% cluster doped photoresist show no metallization at all, whereas the sample of structures fabricated with 1 wt% clusters show gold particles on the polymer and the glass surface as well.

The best results are achieved by ascorbic acid as a mild reducing agent. There are some gold particles attached to the structures whereas the area surrounding the polymers is nearly free of those particles.

Further experiments on metallization of functionalized polymer structures will be carried out by changing the solvent (using water instead of ethanol) and eventually coating the glass slides subsequent to polymerization.
6 Conclusion

The presented diploma thesis describes a method for the direct fabrication of functionalized polymer structures via multiphoton and stimulated emission depletion (STED) lithography.

The functionalization of the two and three dimensional structures is enabled by metal oxo clusters with methacrylate and mercapto groups as ligands, mixed with PETA/DETC, which is known as a photoresist suitable for STED lithography [48, 49]. The incorporation of metal oxo clusters allows for fabrication of polymers with improved properties [52, 53]. By exchanging parts of the clusters’ methacrylate ligands with mercapto groups gives functionalized polymers, which can be useful in fabrication of structures capable to bind proteins or metals.

To our best knowledge, cluster based resins were not applied in STED lithography before.

For determination of the minimum feature size of zirconium-cluster doped polymer structures, lines using multiphoton and STED lithography were written. The smallest features fabricated using a photoresist doped with 2.5 wt% clusters (ZrSH2) are about 70 nm in size.

In order to gain information about the depletion efficiency of different photoresists (doped with varying cluster and mercapto concentrations), depletion test patterns were written. Thereby lines were fabricated, where the excitation power (@ 780 nm) is held constant while the depletion power (@ 532 nm) is increased. This results in decreasing line heights due to stimulated emission depletion until a certain value. Further increasing of the depletion power results in increasing line height, most likely due to residual absorption of the depletion beam.

The data obtained from the experiments show that lower cluster concentrations within the photoresist allow for fabricating smaller features (compared to higher cluster concentrations). The concentration of mercapto groups as ligands for the cluster has an influence on the structure size, too. Doping of the photoresist with clusters carrying higher amounts of thiol groups decrease the depletion efficiency of the photoresist.

Labeling of the structures with Alexa647-maleimide linkers enables investigation of the polymer structure functionalization. The maleimide side of the linker thereby binds covalently to the structure. The Alexa647 signal is obtained via scanning fluorescence microscopy. The fluorescence signal from functionalized structures happens to be
thirtyfold increased compared to unfunctionalized polymers. Therefore, the use of clusters within the photoresist enables direct functionalization of polymer structures.

STED lithography in combination with functionality of photoresists can be useful in a great variety of different areas. The two and three dimensional functionalized structures are able to bind proteins, DNA etc. covalently. The produced structures can for example be used in studies of cell behavior. Additionally, the physical and chemical properties of the structures can be changed by metallization of the polymers.

First experiments regarding gold coating of the functionalized structures were done using gold (III) chloride trihydrate reduced by use of three different reducing agents. Comparison shows that best results can be achieved by using ascorbic acid as mild reducing agent.
7 Appendix

7.1 Slide Preparation

The samples for the STED experiments were written on untreated microscope cover slips (MenzelGläser, Braunschweig, Germany) with 0.17 mm thickness.

7.2 Measurement of Point Spread Functions

We used gold nanoparticles (NP) (gold colloid 50 nm, BBInternational, Cardiff, UK) in order to measure the point spread function (PSF) by gathering the backscattered light. Those NPs were mixed with distilled water at a ratio of 1 to 1000. A small amount of this mix is given to the surface of untreated microscope cover slips and then evaporated. After giving a small amount of about 2 µl of immersion oil onto the NPs, another glass slide covers it up and is sealed with ordinary transparent nail polish.

7.3 Titan-Zirconium Clusters

The Ti₄Zr₄O₆(OBu)₄(OMc)₁₆ cluster (TiZr, OBu = butoxid) appears as slightly yellow powder and has a molecular mass of 2305.2 g/mol (see Figure 26).

![Figure 26: Image of the titan-zirconium cluster (TiZr). a Ti₄Zr₄O₆(OBu)₄(OMc)₁₆ – titanium (blue), zirconium (gold), oxygen (red), methacrylates (grey) b Chemical structure of the butoxid ligand (OBu).](image)
The center of the cluster consists of four zirconium and four titanium atoms. As exchangeable ligands, this cluster has 16 methacrylate ligands, which are used within the polymerization process. These ligands can be exchanged by thiol groups. Two different functionalized clusters where used within the experiments:

- **TiZrSH1**: 34% of the methacrylate ligands have been exchanged with thiol groups. This are, on average five thiol groups and eleven remaining methacrylate groups exchanged.

- **TiZrSH2**: 87% of the methacrylates exchanged by thiols, which are on average 14 thiol groups and two methacrylate ligands for polymerization.
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