SUPER-RESOLUTION PHOTO "CLICK" CHEMISTRY

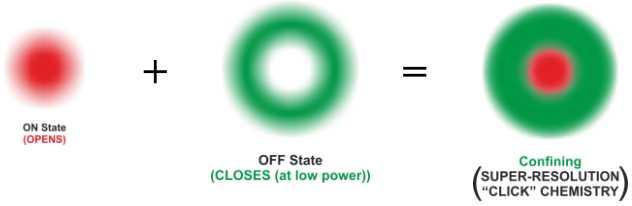
Eljesa Murtezi
International Balkan University, North Macedonia, eljesa.murtezi@ibu.edu.mk

Abstract: The accessible resolution has changed significantly as a result of recent advancements in super-resolution microscopy, which have also significantly overcome the diffraction barrier. A reversible molecular switch serves as the fundamental component, enabling light-promoted activation and deactivation in conjunction with a laser focus that has a regular or tailor-shaped point spread function. Reversible Saturable Optically Linear Fluorescence Transitions RESOLFT microscopy is based on optically switching isomerization states and other optically bistable transitions in fluorophores. For this process, very low laser powers are sufficient for the "depletion" beam since optically triggered transitions of the marker molecule between the two isomeric states are orders of magnitude slower than the fluorescence lifetime. As a matter of fact, the diffraction barrier can be surpassed with considerably lower light intensity of the depletion beam in RESOLFT microscopy compared to STED microscopy.
Recently, this concept has been carried from microscopy to optical lithography. Here we show a system that reaches such a molecular switch for "click" chemistry reactions such as the thiol-Michael addition. In particular, simultaneous irradiation at 532 nm can reversibly deplete the intermediate reversible photo-isomerization state of spirothiopyran (STP) produced by two photon absorption at 780 nm wavelength. This will suppress the subsequent thiol-Michael addition reaction, which forms the chemical basis of the "click" process of maleimide with the thiol. We demonstrate that this mechanism allows for stimulated emission depletion (STED) inspired reversible photoisomerization of STP in order to permit and prohibit the reaction down to the molecular level. STP itself is tested as a photoinitiator for super-resolution optical lithography. A crucial step of this work is the application of RESOLFT for supper resolution tailored chemical reactions, modifications and functionalization. In principle, SPT compounds also give promise as new photoinitiators in the toolbox of STED/RESOLFT optical lithography. The synthesized compounds were characterized using ^{1}H Nuclear Magnetic Resonance (^{1}H NMR), Scanning Electron Microscopy (SEM), and Atomic Force Microscopy (AFM) to confirm their chemical structure, morphology, and surface topography.
Keywords: superresolution, click chemistry, photo chemistry.

1. INTRODUCTION

Examples in effectively enhancing the resolution in optical fluorescence microscopy by reducing the size of the point spread function (PSF) include stimulated emission depletion (STED) microscopy, reversible saturable optically linear fluorescence transitions (RESOLFT), and saturated structured-illumination microscopy (SSIM) (Hell, 2007). In STED microscopy (Hell & Wichmann, 1994) or (Kim et al 2024), fluorescence is inhibited in the outer rim of the PSF by exposing the dye molecules to an additional beam of light, thus pushing the molecules back to the S_0 (ground) state. Instead of quenching fluorophores in the outer rim of the PSF, one can apply the same principle to quench photo-starters of polymerization by STED and hence reduce structure size and enhance resolution in optical lithography as proposed by Klar and Hell in 1999 (Klar & Hell 1999). With features in the nanoscale range, the first experimental proof of STED inspired optical writing (Li et al, 2009) had a significant impact on super-resolution lithography. All super-resolution nano-fabrications have been limited to serial point-by-point patterning using a single focus, which inherently prevents a high quantity of writing over huge volumes, even if the majority of super-resolution writing techniques use high power stimulated emission depletion. The requirement for high laser intensity to saturate the depletion process is the main reason for this constraint. Alternatively, RESOLFT (Hell, 2003) microscopy is based on optically switching isomerization states and other optically bistable shifts in fluorophores. For this process, very low laser powers are sufficient for the "depletion" beam because optically triggered transitions of the marker molecule between the two isomeric states are orders of magnitude slower than the fluorescence lifetime (Hofmann et al, 2005) or (Bossi, et al, 2010) or (Bodén, 2024). Consequently, the diffraction barrier can be overcome with considerably lower light intensity of the depletion beam in RESOLFT microscopy compared to STED microscopy (**Figure 1**). Because of that, massively parallel RESOLFT imaging using 10,000 beams in parallel became possible, allowing for video rate super-resolved imaging (Lefman et al, 2011).

Figure 1. RESOLFT principle



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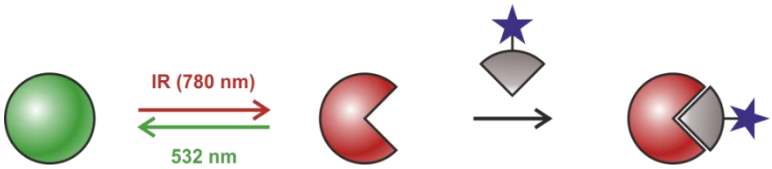
On the way to transfer the principle of RESOLFT microscopy to RESOLFT photochemistry, we herewith intorduce an approach to optically control the photochemistry of molecules by employing photo-isomerization reactions. In particular, we take into account the use of RESOLFT on the thiol-Michael addition process for the reversible photoisiomerization of spirothiopyran (STP), which will provide control over the photochemistry utilizing much lower powers for the saturation of depletion than with conventional STED. Ideally, STP polymers, copolymers and polymer films are prepared. This showed a promising initial indication which led to highly photo-isomerizable STP containing polymers which can be further modified easily through "click" reactions, a well-established concept in chemistry. Green light was found to saturate inhibition of the thiol-Michael addition reaction at relatively low intensity thresholds (Vijayamohanan et al, 2017) or (He et al 2020). By formulating a STP functionalized polymer, we have demonstrated spatial control over thiol-Michael reaction using inhibition by the depletion green light as shown in **Scheme 1**.

Scheme 1. Reaction mechanism of RESOLFT "click" reaction



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Zhu et al. (2015) used photochromism to demonstrate the experimental cross-linking of STP functionalized polymers via the thiol-Michael process, and Boulatov et al. (2016) used mechanochromism. The expectation of exploiting SPT's reversible photoswitching to create the high efficiency inhibition pathway necessary for super resolution writing remains unexplored thus far.

Besides, given the efficient routes of functionalizations through the attachment of functional monolayers or multilayers, photoinduced "click" reactions have been the subject of extensive research in recent years. For this purpose, "click" reactions such as the copper(I) catalyzed alkyne azide cycloaddition (CuAAC) with surface bound alkynes or azides have been applied on a variety surface functionalization, as well as thiol-yne reactions, Diels-Alder and thiol-Michael additions etc. As a result, different methods have been developed over the last years, including metal free ligation chemistry. In this sense, we are in investigation of photoinduced RESOLFT "click" chemistry system which to the best of our knowledge has not been tried yet in super-resolution.

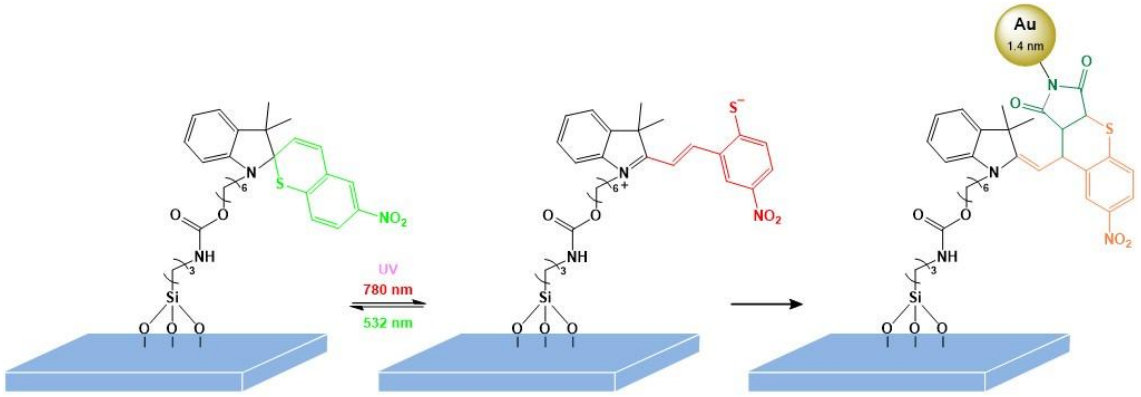
2. MATERIALS AND METHODS

There is a crucial need for a photoresist that is reversibly optically saturable with a low saturation threshold. In this study, we present an early illustration of a chemistry that satisfies these requirements and demonstrate how it can be used for superresolution. We aim to attain super-resolution by using photochromic molecules as an inspiration. Of particular interest is the use of diarylethenes (Majumder et al, 2015). Usually, a photochromic layer of diarylethenes is applied to a resist, and the difference in transmittance between the open and closed photoisomers of diarylethenes is used to incorporate the underlying complement molecule.

We present the covalent integration of a target molecule into a polymer film or backbone that contains photochromic switchable molecules. One of our photochromic system's two photoisomers undergoes a highly selective, high-yielding reaction that "clicks" another molecule or polymer by a thiol-Michael conjugate addition, which is the basis for the writing step in our system. Using photochromic molecules as photoinitiators to reach a low saturable depletion threshold for superresolution lithography is one potential alternate way. Therefore, we focus on STP, a

sulfo derivative of spiropyran, among the photochromic compounds like diarylethenes, spiropyrans, and azobenzenes because one of its two photochromic isomers has a thiolate anion that can be used for thiol-Michael addition chemistry. The "open" and "closed" STP isomers of this molecule alternate photochromically. STP is changed into the merocyanine (MC) isomer by UV radiation, and the MC isomer is changed back into STP by visible (green) light (Shiraishi et al, 2013) or (Yao et al, 2021) or (Sinha et al, 2020). Utilizing the thiolate anion of the MC isomer, which is advantageous as a functional group for further transformations, the suggested STP-based "click" chemical pathway is depicted in **Figure 2**.

Figure 2. Spirothiopyran (STP) photoisomerization induced by RESOLFT, with subsequent "click" reaction.



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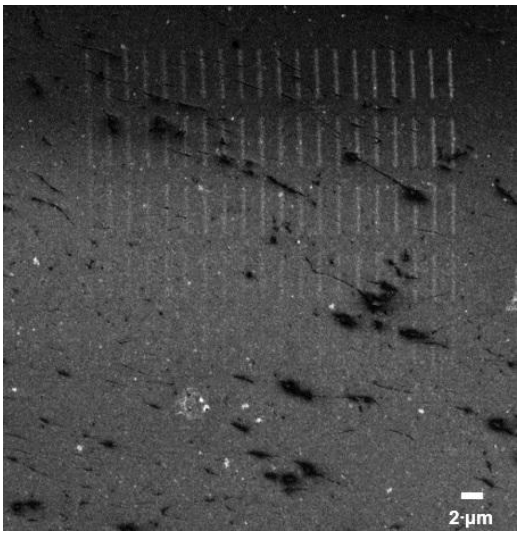
As proof-of-concept, we performed experiments on thermo polymerized films bearing STP on its surface and prove that the MC isomer specifically reacts with the maleimide to form the thiol-Michael addition product under IR (780 nm) initiating two photon excitation (equivalent to the UV wavelength of 390 nm necessary for the STP → MC isomerization) while green light (532 nm) inhibits the addition step through a significant shift in equilibrium toward the nonreactive STP isomer. To achieve low-intensity thresholds for depletion, the photochromic switching must take place on a time scale that is faster than the Michael addition reaction. This would entail resolving technical issues such guaranteeing superior super-resolution control of lithography and functionalization (**Image 1)**.

Image 1. SEM (gold sputtered) image of the gold maleimide functionalization after photoisomerization using 780 nm excitation and increasing 532 nm depletion beam (from right to left hand side) accordingly.



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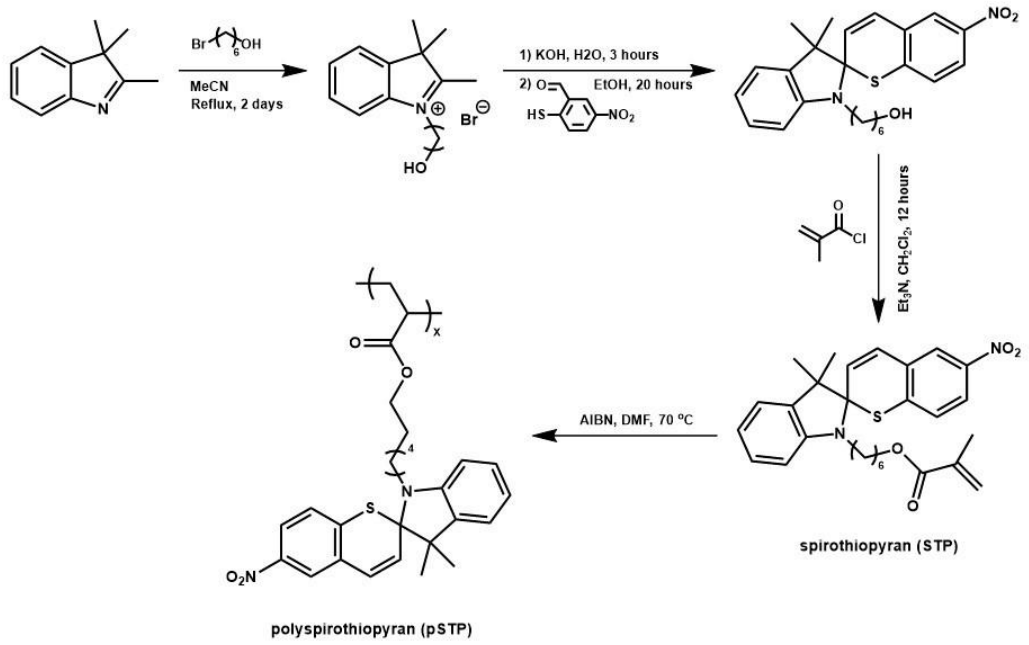
3. RESULTS AND DISCUSSION

During the first phase, STP methacrylate monomers are synthesized and characterized ^{1}H NMR (**Figure 3** and **Figure 4**). Thereby different routes which are described above are employed and tested for their applicability and the most feasible selected to generate robust STP-bearing polymer films (poly-STP). The reactivity of MC with maleimide-functionalized read out fluorophores is tested on a large area (without nanopatterning).

Figure 3. Synthesis route and preparation strategy to polyspirothiopyran (pSTP) starting from 2,3,3-trimethyl-3H-indole.



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The close (STP) and the open (MC) isomers are optically characterized to fit two photon excitation wavelengths and depletion wavelengths. Due to "solvatochromism" effect, the poly-STP (and poly-MC) might have shifted spectra. If so, either modified polymer backbones inducing less chromatic shifts have to be used or different STPs need to be tested. This establishes a "quality-feed-back-loop". The hybrid materials which are synthesized and look most promising in the optical characterization are used for nanopatterning experiments. Special emphasis is given to photoisomerization and click reactions, for instance with maleimide (**Figure 2**), which carries a read-out fluorophore. Other click reactions such as Diels Alder are tested as well. We have so far used only two-dimensional donuts (rings) for 2 dimensional nanopatterning.

Finally, the RESOLFT photo-"click" patterns are characterized by SEM (**Image 1**), microscopy and AFM (**Figure 5**) are used in order to determine the achieved structure sizes and the resolution between two closely spaced structures in the future. Suitable contrast agents have to be found the respective technique. In case of STED and / or STORM microscopy, suitable dye molecules need to be attached to the maleimide. For SEM, gold nanoparticles will probably be the right choice. In case of AFM, the maleimide might be attached to gold nanopartciles, as well, or bulky proteins might give good contrast.

Figure 4. ^{1}H NMR spectra of pSTP in Chloroform ($CDCl_3$).

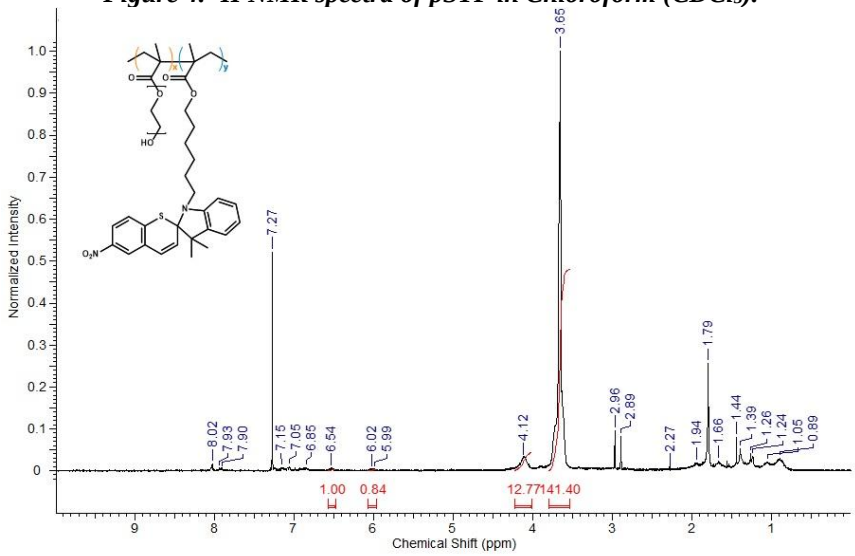

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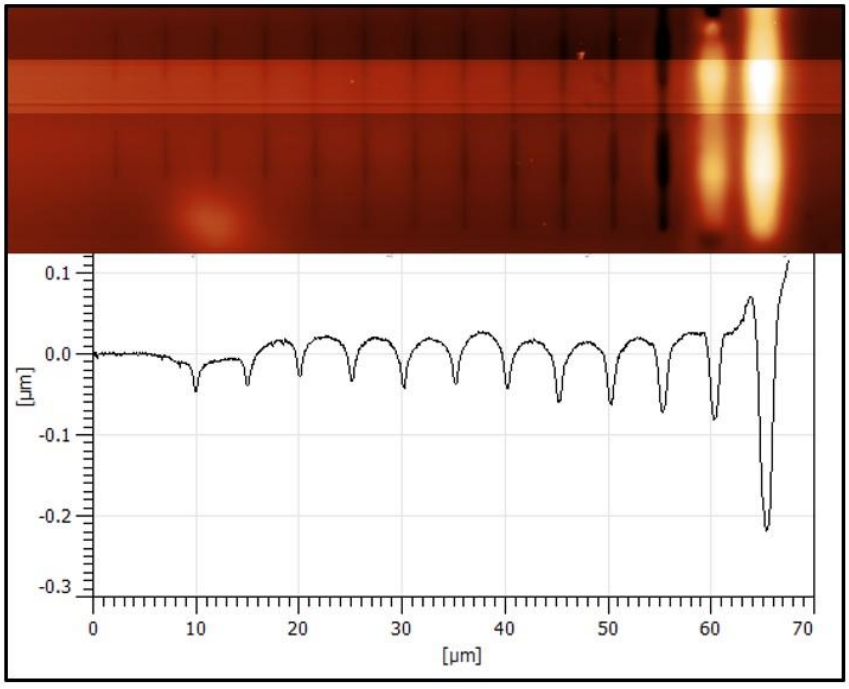
Figure 5. AFM image and its line profile showing the depletion of the isomerisation as a function of 0, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1,8, 2, 3, mW (form left to right hand side) 532 nm depletion wave. The excitaion wavelength was kept constant at 2 mW (780 nm).



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4. CONCLUSION

In conclusion, this pawes the way for the foundation of a superresolution control over the photochemical reactions that uses RESOLFT to integrate thiol-Michael addition chemistry with photochromic switching. These experiments establish the groundwork and indicate the first steps toward realizing a highly correlated photochemical control and fabrication technique with nanoscale resolution. In principle, the photochromic molecules' low saturation thresholds and quick reversible switching make them excellent choices for creating a parallelized subdiffraction 3D writing

system. Using a STP functionalized polymer, spatial control of cross-linking is demonstrated by utilizing photoswitching and thiol-Michael addition chemistry with advantageous kinetics.
The use of RESOLFT for supper resolution customized customization and functionalization is a key goal of this effort. In principle, SPT compounds also give promise as new photoinitiators in the toolbox of STED and RESOLFT for the fabrication of nanostructures. This work would overcome the technical difficulties such as ensuring high quality super-resolution control of photochemistry.

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