A novel approach for near-field optical microscopy based on tip-enhanced fluorescence via plasmon resonance energy transfer (TEFPLASNOM)

Stefan G. Stanciu,[1*] Denis E. Tranca, [1] Giulia Zampini,[2] Giulia Quaglia,[2] George A. Stanciu,[1] Radu Hristu,[1] Fang Yang,[3] Aiguo Wu,[3] Loredana Latterini[2]

[1]Center for Microscopy-Microanalysis and Information Processing, Politehnica University of Bucharest, 313 Splaiul Independentei, 060042, Bucharest, Romania;
[2] Department of Chemistry, Biology and Biotechnology, University of Perugia, Via Elce di sotto, 8 06123 Perugia - Italy
[3] Cixi Institute of Biomedical Engineering, CAS Key Laboratory of Magnetic Materials and Devices, & Key Laboratory of Additive Manufacturing Materials of Zhejiang Province, Ningbo Institute of Materials Technology and Engineering, Chinese Academy of Sciences, Ningbo 315201, P.R. China
*Corresponding authors: stefan.g.stanciu@upb.ro; loredana.laterini@unipg.it

Published: September 8, 2020

ABSTRACT

Microscopy techniques based on tip-enhanced optical processes have gained massive interest lately given their possibility to map the optical properties of single objects and structured surfaces at nanoscale resolutions, and beyond. Among these, Tip-enhanced Fluorescence Near-field Optical Microscopy (TEFSNOM) has been demonstrated as a valuable tool to probe the fluorescent properties of samples based on the complex effects that can be achieved by placing a metallic nanosized structure (the tip) in the proximity of an object that fluoresces. The TEFPLASNOM project is aimed at developing a system enabling a novel TEFSNOM variant that harnesses the plasmon resonance energy transfer from an excited noble metal tip (donor) to a nanosized fluorescent object (acceptor), to achieve enhanced sensitivity, and hence better acquisitions times and resolution compared to conventional TEFSNOM approaches.

Keywords: Tip-Enhanced Near-Field Optical Microscopy, Tip-Enhanced Fluorescence, Plasmon Resonance Energy Transfer.

1. INTRODUCTION

Chemical and structural imaging with nano resolution under ambient conditions are of utmost importance for advancing our current understanding of biological species and processes. Furthermore, comprehending in detail the optical properties of advanced nanomaterials is a critical requirement to improve their design and performances, and to enable efficient functionalization strategies for various real-life applications, e.g. drug delivery, bioimaging, theranostics, etc.

This reality motived over the past decades a consistent body of work in the field of nanoscale imaging, which led to the establishment of a wide variety of techniques responsible for unprecedented possibilities for high-resolution optical imaging. Although far-field nanoscopy techniques based on super-resolved fluorescence (SRM) have generated huge impact in life sciences, they are biased by important limitations, linked to the requirement of very specialized fluorescent probes, or high light exposure levels that can be linked to cyto- and phototoxicity, or photodamage, and other disadvantages[1]. The requirement of very specialized contrast agents also impedes the use of such SRM techniques in materials sciences to investigate important aspects, e.g. the behaviour of photoluminescent nanostructures, compositional inhomogeneities and defects in fluorescent nanomaterials, etc.

Microscopy techniques based on tip-enhancement effects[2-4] are currently regarded with very high interest, as they can overcome some of the most important limitations of SRM techniques, and extract optical properties at nanoscale resolution in ambient conditions and using low power excitation conditions. Landmark experiments have shown that these techniques can be harnessed to yield resolutions even below 1nm [5,6] (in special conditions). The value of Tip-enhanced Fluorescence Near-field Optical Microscopy (TEFSNOM)[7] derives from its underlying contrast mechanism that allows probing the fluorescence of an investigated sample at resolutions depending solely on the dimension of the tip and the sensitivity of the detector. In brief, TEFSNOM exploits the effects of placing a metallic structure in the proximity of an object that fluoresces: modification of its radiative and non-radiative rates, which induce changes in the fluorescence emission intensity and lifetime. The detection concept of TEFSNOM consists thus in the periodic perturbation of the emitted fluorescence using a nanosized (metallic) tip.

TEFPLASNOM's main objective is to demonstrate a novel TEFSNOM variant, carrying the same name as the project acronym, that massively exceeds current approaches in terms of sensitivity, speed and resolution, by exploiting plasmon resonance energy transfer (PRET)[8] from a (noble) metal tip to the fluorescent object of interest.

2. STATE OF THE ART

Although TEFSNOM lacks the optical sectioning capabilities of fluorescence based far-field SRM techniques, its importance is nonetheless tremendous as it does not require high laser beam power intensities, and can be used to investigate at nanoscale resolution any type of fluorescent sample ranging from biological species[9,10], to nanostructured materials[11,12], taking advantage of simple illumination configurations. One of the earliest successes in this field was obtained by Sánchez et. al., who demonstrated the enhancement of two-photon absorption in the near-field[13], resulting in imaging of photosynthetic membranes and J-aggregates with ~20nm spatial resolution. Later, Ma et al.[9] focused their attention on DNA, imaging isolated Cy3 molecules and Cy3 molecule pairs. They achieved an average resolution of 8.2nm when investigating 211 isolated single Cy3 molecules, which they claimed to be superior to the resolution achievable with Atomic Force Microscopy (AFM) using the same probe, given that the force involved in AFM imaging is proportional to the inverse of the tip-sample distance and decays much more slowly than the optical near field. In a more recent experiment presented by Schulz et al.[10], the authors used an AFM setup combined with a confocal fluorescence lifetime microscope to exploit localized fluorescence quenching of organic fluorophores by means of a sharp n-doped silicon tip. This effect was addressed both spatially and temporally to achieve correlated optical and topographic images at resolutions <5 nm. They demonstrated their approach on individual Atto655 molecules and on DNA origami triangles with 120 nm sides into which two fluorophores were incorporated at 20.7 nm from each other, a theoretic localization confirmed by their tip-enhanced fluorescence approach. Such previous experiments have thus demonstrated the huge resolution potential of TEFSNOM, but its widespread is still severely limited by the high background, which interferes with collecting the signals of interest. Furthermore, either with setups in reflection [14] or transmission[15], interferences between the fluorescence of the sample directly measured by the detector and the near-field fluorescence of the same excitation area, scattered by the tip before reaching the detector, have been reported. Thus, even though TEFSNOM enables superb optical resolutions, high background, and unwanted effects (e.g. interference patterns), difficult to control, make practical imaging applications cumbersome. Low signal-to-noise ratio imposes high integration times, hence low acquisition speeds are required to collect meaningful images.

3. BREAKTHROUGH CHARACTER OF THE PROJECT

This project devotes to overcoming the above-mentioned limitations of traditional TEFSNOM, by exploiting Plasmon Resonance Energy Transfer (PRET)[8] to generate fluorescence[16]. Related strategies have been successfully used so far in plasmon-enhanced spectroscopy approaches[17]. In the novel imaging modality, coined TEFPLASNOM, same as the project's acronym, instead of exciting the fluorophore directly using photons of frequency within its absorption spectra, fluorescence emission is achieved via PRET from the metallic tip that is scanned across the sample area. This results in the significant elimination of background signals coming from neighbor fluorescent molecules in the proximity of those that are imaged, resulting in massively higher sensitivity, acquisition speed, and resolution.

Furthermore, this project also aims to augment the fact that complementary microscopy techniques based on tip-enhanced optical processes can be operated in-tandem, in correlative approaches, resulting in spatially (and temporally) co-localized information. For example, in a recent correlative imaging approach, Meng et al.[18] theoretically proposed a nano plasmonic strategy for precision in-situ measurements of TERS and TEFSNOM, which holds potential for becoming a popular approach for spectral analysis at nanoscale. To better exploit the fact that TEFSNOM used in combination with complementary techniques can provide a more complete picture of the physico-chemical properties exhibited at nanoscale by a sample of interest, we develop a TEFSNOM/TEFPLASNOM setup that takes the form of a module that can upgrade s-SNOM systems, s-SNOM[3] being one of the most popular techniques based on tip-enhancement. This will facilitate the widespread of tip-enhanced fluorescence imaging, given the increasingly large number of s-SNOM systems worldwide, and the superb complementarity of the two contrast mechanisms.

4. PROJECT RESULTS

To achieve the set objectives, we have focused on two research & development tracks: optical systems engineering and materials engineering. In the first research track, a commercial s-SNOM system equipped for IR, neaSNOM (Neaspec Gmbh, Germany), was custom modified to incorporate an additional (external) module that allows performing TEFSNOM imaging in the visible based on conventional and PRET approaches. This module, Fig 1, was designed so as to allow both imaging and spectroscopic assays, by incorporating a free space lock-in coupled adjustable silicon optical receiver with sensitivity in the range of 350nm-1100nm, 205-FS (Newport), for imaging tip-enhanced fluorescence, and a Flame S-VIS-NIR spectrometer (Ocean Optics), which allows to spectrally resolve the optical signals of interest (e.g. related to the probe, sample or substrate). Two illumination options were made available: (i) fixed wavelength, corresponding to a CW beam (633nm now available, 488 nm and 532 nm lines to be added); (ii) tunable wavelength, available with a supercontinuum (SC) pulsed laser, SuperK Compact (NKT Photonics),

450-2400nm, coupled to an AOTF based optical filter, tunable between 450-700nm, with 2,5 – 8,5nm bandwidth. The beams originating from these sources are inserted to the optical path of the neaSNOM system and directed to a parabolic mirror that focuses them on the tip.

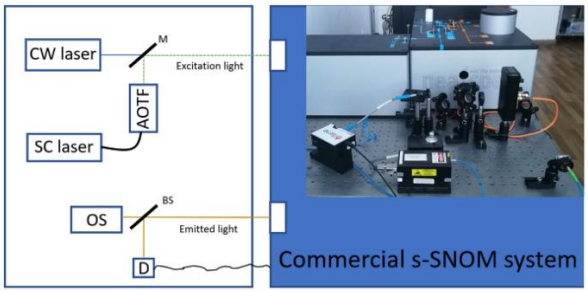

Fig 1. Schematic representation of the implemented setup for conventional and plasmon resonance based TEFSNOM imaging developed to extend a commercial s-SNOM system: a retractable mirror is used to switch between CW and pulsed SC illumination. An AOTF based tunable filter is used to select the wavelength required to promote plasmon resonances in the tip, for TEFPLASNOM imaging. A beamsplitter is used to direct the light emitted by the sample towards an optical spectrometer, and to a free space optical receiver, coupled to the lock-in amplification scheme of the s-SNOM system. Inset: Photo of optical setup.

In the first implemented imaging experiments we have addressed very bright samples, consisting in commercially available fluorescent quantum dots, Qdot 655 (Thermo Fischer), which were imaged with conventional TEFSNOM under CW illumination. Additionally, we have extended our efforts towards exploring whether the TEFSNOM setup is also suitable for performing studies on upconversion fluorescence (namely excitation in IR and emission in the visible). For this, we have used custom developed NaYF4(Er15%) nanoparticles (NPs) that we excited with 1550nm (CW line available in the commercial s-SNOM system), to observe their emission in the visible, Fig. 2. The upconversion emission peaks of these NPs under excitation with 1550nm are located at 520nm, 550nm, 650nm, 980nm, in the range of the detector available in our setup.

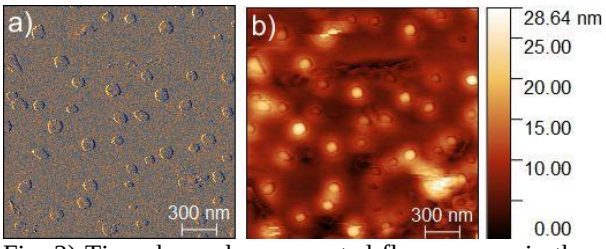

Fig. 2) Tip-enhanced upconverted fluorescence in the visible (a) of NaYF4(Er15%) nanomaterials (b, topographic image) under excitation with 1550nm.

The TEFPLASNOM workmode, still under development, requires illuminating a noble-metal coated tip with a wavelength that promotes plasmon resonances. In case of samples where the plasmon resonance spectra overlaps with the absorption band of a fluorophore, the latter will emit based on the PRET phenomena. In case the optical properties (absorption band) of the tip are *a priori* known, the AOTF can be tuned from the start to enable illumination with the required wavelength. Alternatively, in case the optical properties of the tip are not known the AOTF can be incrementally tuned so that the illumination light is swept across a wavelength range where plasmon resonances can be achieved, and an illumination "sweet-spot" can be identified either by measuring the absorption of the tip, or by assessing the fluorescence yield of the sample. This second modality is of course more laborious, and the first would be preferred, however, commercially available AFM tips are not accompanied by specifications of their optical properties. Therefore, in the frame of the project, we have commenced developing a strategy for custom fabricating tips for TEFPLASNOM imaging, with known absorption properties. For this we designed a fabrication procedure, which is currently being optimized. In brief, a tipless cantilever, HQ:NSC35/tipless/Cr-Au (MikroMasch), is initially cleaned, activated, and functionalized. Further on, AuNPs with known optical properties are attached to the cantilever, to act as a tip. Another important development stage, pending to be completed, is connected to the fact that in TEFPLASNOM imaging under illumination with a pulsed beam originating from a SC laser, the optical signal of interest is modulated by both the oscillating tip and the laser pulse frequency, adding extra difficulty to the problem of lock-in extraction of tip-enhanced signals, compared to conventional TEFSNOM. The complexity of this problem is also closely intertwined with the relationship between the two modulation frequencies, that of the tapping tip, and that of the pulsed excitation beam[19].

Preliminary results on conventional TEFSNOM imaging are used as reference in the work addressing the second research track of the project dealing with materials engineering. In brief, a main objective of this research track consists in the design and synthesis of samples that are suited for demonstrating the advantages of TEFPLASNOM imaging in terms of resolution and sensitivity, and on identifying the materials that are best compatible with the available experimental settings. After careful assessment of various options, we have decided to prepare dye-doped silica nanoparticles as samples, and we adopted two types of fabrication procedures. In the first, compact silica nanoparticles (CSN), were synthesized according to a previously reported procedure, that was custom modified[20,21]. Further on these instances were amino-surface functionalized (CSN-NH_2), and used for the subsequent adsorption of Protoporphyrin IX and Cytochrome C dyes; these dyes have been selected because they have absorption transitions in the Vis region, where plasmonic absorption of gold colloids is expected. Near-field optical responses of these samples have been investigated with a novel method for quantitative analysis

of s-SNOM data based on phasorial representations[22], whose development was partially supported by this project. In a second fabrication procedure that we designed and developed, mesoporous silica nanoparticles (MSN) were synthesised according to a previously reported procedure[21,23]. The MSN samples were used as templating material for the adsorption of Fluorescein (FITC) and Nile Blue (NB); these dyes are selected because the fluorescence spectrum of FITC overlaps with plasmonic band of gold, while NB fluorescence is at lower frequencies compared to plasmonic band. In brief, the MSN-FITC sample was prepared through a co-condensation method, wherein the fluorophore is added during the formation of the silica network, while the MSN-NB samples assembled by exploiting the electrostatic interaction between the negatively charged MSN and positively charged Nile Blue (NB). The optical properties of these materials have been characterized with a variety of optical characterization methods and currently await investigations with the forthcoming TEFPLASNOM workmode.

A second main objective of the materials engineering track concerns the better understanding of the PRET[8] phenomena, so that connected TEFPLASNOM developments can be better optimized. For this a framework for investigating various aspects concerning PRET from an AuNP towards a fluorophore was designed. Custom AuNPs were fabricated with a multi-steps approach that relies on the synthesis of small AuNPs which act as "seeds" for the subsequent growth of gold shell[24]. This growth procedure was iterated to obtain AuNPs (Fig. 3) of the desired size. Further on, the obtained AuNPs samples were coated with polymeric shells that embedded NB. This coating strategy was based on a step-by-step process, wherein PSS and PDDA polymeric layers were added sequentially[25], to control the thickness of the polymeric shells around the AuNPs and the distance between the plasmonic surface and the dye. Au plasmons and Dynamic Light Scattering (DLS) measurements (Fig. 3) showed that the growth of different polymeric shells was successfully achieved. At present we are assessing via spectroscopic methods the influence of various illumination wavelengths falling in the absorption band of the AuNPs over the fluorescence yield of the NB fluorophore embedded in the shell, in the purpose of identifying how the donor – acceptor distance influences the efficiency of the PRET phenomena, an aspect that is not yet exactly understood.

5. FUTURE PROJECT VISION

5.1. Technology Scaling

We envision that Phase 2 of Attract would allow taking the demonstrated technology further, at the borderline of TRL7: TRL 7 – "system prototype demonstration in operational environment" and TRL 8: TRL 8 –"system complete and qualified". The main steps required to scale up the proposed technology, namely a module for TEFPLASNOM imaging, deployable as an upgrade to commercial s-SNOM (or AFM) systems, are:

- Development of fabrication procedures for low-cost, ultra-sharp, robust tips with known optical properties.
- Development of highly efficient strategies for lock-in extraction of double modulated tip-enhanced optical signals.
- Development of algorithms for automated identification of the plasmon resonance band in commercial noble metal based tips.
- Development of artificial intelligence methods based on generative adversarial networks for improvement of resolution and speed[26], tuned to the specifics of TEFPLASNOM imaging.
- Demonstration of the functionality and usefulness of the envisioned TEFPLASNOM system in life and materials sciences labs (end-users).

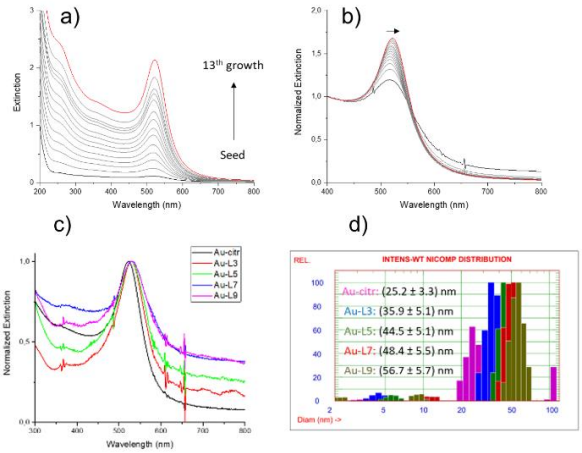

Fig. 3. Assessment of optical properties of the designed AuNPs and polymeric-shell coated AuNPs, custom designed to better understand the PRET phenomena. (a) Extinction and (b) normalized extinction spectra (at 400 nm) of AuNPs seeds and growth steps, (c) normalized extinction spectra and (d) DLS measurements of Au-polymeric shell after several layer depositions procedure.

5.2. Project Synergies and Outreach

An optimal consortium configuration for scaling up the TEFPLASNOM technology in a potential Attract Phase-2 endeavour would require (besides the two initial partners, CO: UPB and P1: UNIPG, and the third institution authoring this study - with expertise in nanobioimaging):

P3: SME partner dealing with fabrication of probes for AFM and tip-enhanced microscopies; P4: SME partner involved in the development of systems for AFM or tip-enhanced optical imaging; P5: academic/SME partner with expertise in generative adversarial networks; P6/P7: Two end-user labs with expertise in the biology of

eukaryotic/prokaryotic organisms; P7: An SME partner with expertise in business management.

To augment the impact of the developed technology and accelerate the scaling up, outreach activities will represent an important focus. We intend to liaise with important EU initiatives, such as the Euro Bio-Imaging Network, European Materials Characterisation Council (EMCC), or ongoing COST Actions implemented by stakeholders in the involved scientific domains (nanoimaging, advanced materials, life sciences). Via these dialogues, we will identify EU groups in need of the developed technology and implement proof-of-concept experiments together with them to showcase how the developed technology can enable new research avenues. Identification of funding avenues for augmenting the outputs of TEFPLASNOM Phase 2 will also be at focus.

5.3. Technology application and demonstration cases

In a potential Phase 2 project we envision the participation of four end-user labs that will facilitate the system prototype demonstration in operational environment (TRL 7). Two-end user labs with expertise in advanced materials will demonstrate the usefulness of the proposed imaging system for characterizing optical properties nanobiomaterials to enable/facilitate their functionalization for nanomedicine. Two end-user labs with expertise in the biology of eukaryotic and prokaryotic organisms will demonstrate the usefulness of the proposed technology with respect to enabling novel fundamental research routes that can sched light on still unresolved aspects such as the drug-resistance mechanism of bacteria, or nuclear envelope reassembly; the technology will also give fundamental information on cell proliferation and differentiation, which are essential in cancer diagnosis or in tissue engineering. The four end-users will be in close dialogue to also develop joint applications for bioimaging, and theranostics, that build on the novel knowledge enabled by TEFPLASNOM.

5.4. Technology commercialization

During the lifespan of the Phase 2 extension of TEFPLASNOM, a detailed business plan will be put in place, which is required for a swift translation of the TRL7-8 system to the market. Intellectual Properties (IPs) developed in the project will be harnessed to keep-on top of competitors. They will also help efficiently monetize the gained know-how by enabling IP licensing routes. As mentioned in 5.2 a partner with expertise in business management is planned to be included in the consortium.

5.5. Envisioned risks

The ambitious goal of scaling up the developed technology will need to cope with the important risks associated to commercial and scientific competition. Furthermore, given the high interdisciplinary nature of the project, the expected duration of the implementation period could also pose a risk, as it takes time to establish fluent workflows for efficiently running joint research and development activities by groups with different backgrounds.

5.6 Liaison with Student Teams and Socio-Economic Study

TEFPLASNOM Phase 2 will nominate an experienced person to liaise with relevant MSc. level student teams, to familiarize them with the developed technology and its potential for advancing various fields of research. Furthermore, SME partners participating in this project will create internship positions for MSC students, and academic partners will create PhD positions for MSc students in their final year to consider. To contribute to the expert-driven socio-economic study that will be organized by ATTRACT, TEFPLASNOM Phase 2 will make available all required information, and everyone involved in the project will commit time to participating in interviews, polls, etc.

6. ACKNOWLEDGEMENT

Work with the neaSNOM (neaSpec) system was possible due to European Regional Development Fund, Competitiveness Operational Program 2014-2020, Project No. P_36_611, MySMIS code 107066, INOVABIOMED. The contribution of the Cixi Institute of Biomedical Engineering in China was possible via the support of the support of the National Natural Science Foundation of China (51803228) and the Ningbo Natural Science Foundation of China (2019A610192). This project has received funding from the ATTRACT project funded by the EC under Grant Agreement 777222.

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