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Metasurface-based Total Internal Reflection Microscopy

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Abstract: In recent years there has been a significant effort to develop dielectric metasurfaces for visible light applications allowing arbitrary wavefront manipulation using ultra-thin optical devices. In the context of bioimaging, one technique particularly suited for the study of molecules near a cell membrane is Total Internal Reflection Fluorescence Microscopy(TIRFM), generally implemented using bulky high-NA, small field-of-view (FOV) oil objectives. We present the design, fabrication and experimental validation of metasurface-based TIRFM substrates consisting of periodic 2D arrays of asymmetric structures fabricated in TiO₂ on borosilicate glass. These metasurfaces have been measured to couple up to 70% of the transmitted light into the first diffraction order at 65° in glass achieving total internal reflection when reaching sample's aqueous media, eliminating the need for oil objectives or prisms, while having a direct transmission efficiency under 7% to minimize spurious fluorescence. This simple-to-use, large FOV technique provide unique TIRF capabilities not accessible by traditional methods, and was used to image immunostained 200 μ m-large bone marrow Human Mesenchymal Stem Cells. We foresee these kind of metasurfaces becoming valuable tools to incorporate TIRFM's high contrast, low photodamage and low photobleaching capabilities to inexpensive wide-field microscopes.

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1. Introduction

During the last three decades, total internal reflection fluorescence (TIRF) microscopy has been the preferred choice among the very few commercially available high axial resolution optical microscopy techniques, due to its relative simplicity and virtually unrivaled performance for the study of cell membrane dynamics. TIRF systems, largely based on high numerical aperture (NA) oil objectives, rely on an evanescent field merely touching the observed sample to selectively excite those regions of interest, with no light reaching the bulk of the sample and hence significantly improving image contrast while minimizing photobleaching and photodamage. This objective-based approach provided several advantages over the preceding prism-based TIRF microscopes, but not without also incorporating its own set of limitations, such the need of direct physical contact with the sample, a small maximum field-of-view (FOV) or the intrinsic cost of those objectives, among the most expensive of all commercially available optical microscopy objectives.

On the other side, in recent years there has been a thriving development of a new generation of optical components, expected to have a major impact on a wide range of next-generation of optical systems: Metasurfaces (MSs), the bidimensional counterpart of so-called metamaterials, typically consists of periodic or quasi-periodic arrays of nanoresonators designed to impart a highly localized and arbitrary phase point to point across the area of the MSs, allowing a complete customization of the transmitted or reflected wavefront profile. Differently to traditional refractive optical components where the changes on the propagating light gradually accumulate over

relatively large distances, MSs offer an abrupt change on the beam's properties, generally within a distance shorter than one wavelength. This unique characteristic along with the possibility to achieve unconventional behaviors that have never existed on traditional optical components, has proved to be useful for a wide range of applications from spectroscopy [1] to integrated optics [2], optical filtering [3], image sensors [4], holography [5,6] and biosensors [7].

Specifically in the context of optical microscopy, modern metasurfaces adapted for the visible spectrum will most definitely have a major impact, despite the relative scarce work currently aimed directly to this application. Aside from the impressive ongoing progress on the development of achromatic metalenses, whose scope of application goes well beyond microscopy, limited work can be mentioned. Some notable recent exceptions are the use of MS to improve axial resolution on laser scanning microscopy [8], metalenses for two-photon microscopy [9], the improvement of fluorescence microscopy image contrast using resonant structures [10] and the removal of the orientation-induced localization bias on single molecule microscopy [11].

Beam steering has been among the first pursued objectives in all MS developments, usually as an intermediate step necessary to later target more complex responses (e.g. focusing), with some of the first experimental realizations dating back to 1998 [12]. While the efficiency and complexity of these beam steering MSs has increased significantly [13–16] with the advent of more advanced design tools (e.g. inverse design algorithms [14–16]) and nanofabrication technologies, the full implementation of MS-based TIRF microscopy technique has never been realized nor proposed.

The TIRF device conceived in this paper consists on a special substrate that does not require any particular high-NA objective to achieve the excitation beam's total internal reflection and evanescent field. Instead, a purposely designed MS grating redirects most of the free space incoming laser energy into one of the first diffraction orders, acting as a blazed grating light coupler for inexpensive, large FOV TIRF microscopy. We experimentally demonstrate these MSs can couple up to 70% of the transmitted light into the first diffraction order at a 65° angle inside the glass substrate, sufficing total internal reflection conditions for a glass-water interface. We finally used these MSs to image immunostained 200 μ m-large bone marrow Human Mesenchymal Stem Cells, typically exceedingly wide to be imaged in a single objective-based TIRF frame.

This paper is organized as follows: in Section 2 we discuss the design, fabrication and characterization of MS-TIRF devices, continued in Section 3 by the results of their use in fluorescence microscopy and bioimaging. Section 4 provides the final conclusions.

2. Metasurface design, fabrication and characterization

In order to conceive a device using only biocompatible materials, transparent in the visible, while maximizing the refractive index contrast between the MS and its medium we chose TiO_2 as the structure's material and borosilicate glass as the substrate. The fabrication process of our metasurfaces is as follows: A 230 nm-thick layer of amorphous TiO_2 is deposited (Plassys MEB 800 IAD e-beam gun system) on a 25x50 mm borosilicate glass. Two positive electron beam resists (A2 PMMA and EL10 MMA) are sequentially spin coated to create a bilayer stack with a higher sensitivity lower section, metalized with 20 nm of aluminium and patterned using an electron beam writer (Raith EBPG-5000+). After a MIBK/IPA (1:3) resist development the samples are rinsed in IP alcohol and a 20 nm nickel layer is deposited (Plassys MEB550SL e-beam evaporator). A lift-off process is realized in a trichloroethylene bath (85°C) followed by a HBr/BCl₃ TPSA-ICP TiO₂ reactive-ion etching. The remaining Ni is finally removed with a 1-min immersion in nitric acid 26%.

For the metasurface's design, we utilized the RCWA software RETICOLO [17] to calculate the diffraction efficiencies and amplitudes of 2D stacked gratings. Starting from a 1D double groove structure (similar to those of [18] and [19]) and using a modified gradient ascent algorithm over hundreds of sets of 9 random initial dimensional parameters defining a generic structure layout,

we converged to the design shown in Figure 1 (a) and (b). For this purpose, we defined and used a figure of merit (FOM) consisting on the ratio between the diffraction efficiency of one of the first orders (-1) and the sum of the other two orders (i.e. 0^{th} and 1^{st}), resulting in an asymmetric composite layout of alternated continuous and segmented lines. The final sample is shown in Figure (d).



Fig. 1. (a) The horizontal geometry of the chosen 2D metasurface design for $\lambda_0 = 640nm$, with $p_X = p_Y = 465nm = 0.73\lambda_0$, $a_W = 160nm$, $b_W = 61nm$, $b_X = 186nm$ and $b_L = 322nm$ and a height h = 230nm along with (b) a scanning electron microscope (SEM) image (45° tilt) of the fabricated sample. (c) When TE-polarized light hits the MS, up to 70% of the transmitted light is coupled into one of the first diffraction orders towards the center of the substrate, 21% is coupled in the opposite first order and only 9% into the traversing zeroth order. (d) The entire 25x50 mm substrate can accommodate multiple MSs, with 40 shown in this sample.

The MSs were characterized using an Olympus UApo N 100X 1.49 oil objective to collect all transmitted diffraction orders from the other side of the 170 μ m glass substrate (the pair of first orders at a 65° angle would be internally reflected without the index-matching oil). The three transmitted beams are hence focused by the objective on its back focal plane (BFP), allowing us to measure their intensities by imaging said plane on a CCD sensor.

When external TE polarized light at $\lambda_0 = 640nm$ hits the MS, which can be situated on either side of the substrate depending on the microscope configuration, we measure 70% of the transmitted light being diffracted into the first order of interest (by design the one directed towards the center of the substrate), 21% is coupled in the opposite first order and only 9% into the traversing zeroth order, as seen in Figure 2. While these are the most relevant values for our specific application in which reflected or absorbed light play a very limited role, we can also measure each diffraction order relative to the incident intensity (absolute diffraction efficiency), in which case the efficiencies are 43%, 13% and 6% respectively. This values closely resemble those efficiencies obtained on the RCWA simulations, of 55%, 16% and 4%, with the remaining 25% being reflected by the MS. The minimal direct transmission of 4% is a key characteristic of any MS design aimed for TIRF microscopy, due to the eventual close proximity between the MS and the stained biological sample and the fact that this beam will never be coupled into the glass substrate, potentially deteriorating the image's contrast if stray light were to reach the observed sample.



Fig. 2. When a TE incident beam hits the (a) plain glass substrate (reference) or (b) the MS, the BFP images captured let us see and measure the transmitted light distribution. By using one of our MSs, up to 70% of the transmitted light is coupled into one diffraction order, that exits the MS at a 65° angle and thus is totally internal reflected in the microscopy substrate's sides.

3. Fluorescence microscopy experimental setup and results

This setup, visible in Figure 3, was based on a X-Y motorized stage with a metasurface holder, an Olympus UPlanSApo 40X NA 0.95 air objective mounted on both a motorized Z stage and a piezo Z-stage (PIFOC, Physik Instrumente GmbH) allowing for fine and course movements, two 637nm continuous wave diode laser systems for single or simultaneous widefield (WF) and TIR illumination (Coherent OBIS 140 mW with a ZET 640/10 Laser Clean-up Filter) and a camera ANDOR iXon Ultra DU-897U-CS0-#BV with a 512x512 pixel sensor (16µm pixel size). The beam reaching the MS was TE polarized with a Thorlabs CM1-PBS251 Polarizing Beamsplitter Cube and finally slightly focused with a Thorlabs AC254-300-A-ML 300mm achromatic doublet.



Fig. 3. The setup used allows to illuminate the sample independently and arbitrarily with a TIR evanescence field or WF illumination using two 637nm continuous-wave lasers. An Olympus UPlanSApo 40X NA 0.95 air objective and an ANDOR iXon Ultra camera are used to image the fluorescent sample mounted on a custom motorized stage. The lens L1 weakly focuses the incoming beam, linearly polarized by P1. TL focuses the MS plane into the CCD.

We proceeded to verify that our metasurfaces could selectively excite fluorophores in close proximity to the substrate's surface, while keeping the bulk of the specimen unaffected, in the dark as is the case with traditional TIRF techniques. For this purpose a highly diluted solution of fluorescent particles (InvitrogenTM FluoSpheresTM Carboxylate-Modified Microspheres, 0.2 µm, dark red fluorescent 660/680 0.0001% in volume) was prepared and used for a simple qualitative TIRF test: A microdoplet of the solution was deposited on the center of the coverslip, followed by the same volume of water to further dilute it, and left to dry fixing some fluorescent particles onto the surface. This was followed by a second, larger (5-10µl) droplet of the solution, obtaining a state in which some particles are fixed to the surface, and a much larger number of particles are diffusing in water with some of them occasionally bumping against the surface. Finally, a series of continuous images were obtained while alternating between widefield illumination, MS-TIRF illumination or both.



Fig. 4. Two frames of an image series before (c) and after (d) switching from WF illumination to MS-TIRF as the only excitation source for the 0.2 μ m Invitrogen FluoSpheres. Integration time: 100ms/frame. While WF allows us to see the rapid movement of the diffusing particles in the bulk of the solution, it also partially hides the fixed particles in focus in the glass surface. MS-TIRF illumination manages to selectively excite those focused microspheres, removing the intense background produced by the diffusing particles. For shorter integration times, the occasionally particle bumping into the surface and blinking for an instant as it passes by the evanescent field is also visible.

Figure 4 (c) and (d) show two frames of a series of 200 frames, each with a 100ms integration time, for a resulting 20 seconds video. With 10 seconds of WF illumination, followed by 10 seconds of MS-TIRF illumination, we observed a clear distinction between a bright and effervescent rapid diffusion activity of the microspheres in the bulk of the solution, followed by an almost static image of those particles fixed to the surface, with a markedly improved contrast of those in-focus FluoSpheres.

While at 100ms integration time per frame on MS-TIRF illumination we rarely saw the rapid event of a free microsphere touching the surface, reducing the integration time let us visualize the 'blinking' effect of particles diffusing down and entering the evanescent field, becoming visible for an instant, to quickly continue their path returning to their dark state.

Finally, by analyzing the MS-TIRF frame in 4(d) we can obtain the maximum and mean pixel value of the bright fluorescent particles and the background noise standard deviation, resulting on a peak-signal-to-noise ratio (PSNR) superior to 700 (the PSNR obtained on a objective-based TIRF image of the sample shown in Figure 5 was 295).

In order to exploit the unusually large field of view offered by MS-TIRF, the biological sample consisted on bone marrow Human Mesenchymal Stem Cells (hMSC-bm) immunostained with anti-Paxillin and anti-rabbit IgG Atto 647N and finally mounted (i.e. encapsulated in aqueous media). This marker will preferably adhere to the cell's focal adhesions, intentionally chosen due to their essential roles in important biological processes including cell motility, cell proliferation, cell differentiation, regulation of gene expression and cell survival. When cultured these cells can reach several hundred microns in size, often well beyond the FOV of TIRF objectives. While fixed cells have been the preferred option for this initial biological long-lasting experiment, no performance difference is to be expected for live samples of the same type.

With the biological sample in place in the MS-TIRF setup, we successfully obtained a series of high-contrast images with the immunostained cells exclusively excited by the MS-generated evanescent field. Similarly to the test with FluoSpheresTM, the observable section of the cells is reduced to only a thin slice in close proximity with the glass surface, greatly reducing the out-of-focus light captured by the air objective. Figure 5(b) is one of such images, where there is a clear intensity distinction between the focal adhesions and the rest of the cell. Despite those specific points having a higher fluorophore concentration, Figure 5(a) show us the existence of an even higher concentration on the cell's nucleus, which is virtually invisible on the MS-TIRF image, letting us see additional information below it.



Fig. 5. Bone marrow Human Mesenchymal Stem Cells stained with Atto 647N (higher marker density of focal adhesions) imaged with (**a**) a standard WF microscope and (**b**) a MS-TIRF substrate. When equalizing the image intensity with the brightness of the zones of interest (top (a) and (b)) the background fluorescence coming from the cell's nucleus hides any information from the membrane below it (center (a) and (b)). For reference, framed in blue is the FOV obtainable with a typical 100X TIRF objective $80 - 100 \,\mu\text{m}$.

The Space-Bandwidth Product (SBP) is a mean to quantify the combination of resolution and field of view of an objective, which can also be translated into the amount of information that it can transmit. The SBP can be expressed as the minimum amount of pixels on a sensor needed to capture all the information provided by the objective's resolution and FOV. It can be easily calculated as the FOV surface divided by the pixel area required to achieve Nyquist sampling at the resolution given by the objective's NA and illumination wavelength. Using the laser source

and objectives described in Figure 3 we calculated a SBP of 2.2 megapixels (100X NA1.49 FN=22 FOV=220 μ m) and 8.2 megapixels (40X NA0.95 FN=26.5 FOV=660 μ m), almost 4 times as much information in favor of the lower magnification objective. This number could climb up to almost 6 by using a water 40X NA 1.2 objective, further reducing the resolution difference with a traditional TIRF objective. Such an enlargement of TIRF's FOV and information bandwidth could be a valuable asset to automate the imaging process of slow-moving or large subjects (e.g. the cells shown in this work), as well for biopsies of large specimens.

4. Conclusion

In this work we introduced the concept of MS-TIRF microscopy. We designed, fabricated and experimentally tested TiO_2 metasurface gratings capable of coupling as much as 70% of the transmitted light into one first diffraction order, while greatly suppressing the direct transmission, both important conditions for a powerful TIRF coupling device. We used fluorescent microspheres to verify MS-TIRF's selective illumination and measure the resulting signal-to-noise ratio, resulting in more than double that of a similar objective-based TIRF image. Finally, we imaged immunostained Human Mesenchymal Stem Cells using our MS-TIRF device and a 40X air objective, obtaining very high image contrast over a FOV 9 times larger than that of traditional TIRF systems. The calculated SBP is also higher by a factor of 4. In conclusion, we showed that TiO_2 metasurface substrates provide a powerful alternative to high-NA objectives that expand the capabilities of modern TIRF microscopy. The wide flexibility of MS design could also allow for more advanced design features that tackle common weaknesses of TIRF imaging such as the homogeneity of the excitation field further improving the edge of MS-TIRF over existing techniques.

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Disclosures

The authors declare no conflicts of interest.

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