

Evanescent-Field Excitation of Fluophores in Cultured Neural Networks by Integrated Polymer Waveguides

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Abstract—Monitoring the interface between adherent cells and their respective substrate using fluorescent methods traditionally depends on complex systems not suitable for all types of cell studies. We present a method for cell–substrate monitoring by optical waveguides fabricated using deep UV (DUV) modification of polymethyl methacrylate. This simple process allows neuronal cells to be grown directly on optical waveguides in a more conventional, *in vitro* environment, similar to cells grown in Petri dishes. By using the evanescent field generated at the interface between the waveguides and cells, fluorescent molecules present in the cells can be excited within several hundred nanometers of the optical waveguides. Details on the DUV chemistry and fabrication technologies will be briefly outlined as well as proof-of-concept of fluorescent excitation using the evanescent field generated by optical waveguides.

Index Terms—Deep UV (DUV), evanescent field, neuron, polymethyl methacrylate (PMMA), total internal reflection (TIR), waveguide.

I. INTRODUCTION

AS THE study of cellular processes advances, new techniques for monitoring important biological factors are becoming increasingly important. These new tools are developed at the interface between biology, chemistry, and engineering. In particular, micro- and nanofabricated systems are beginning to offer insights into cellular mechanisms previously unable to be probed with such great sensitivity [1], [2]. Optical waveguides offer noninvasive methods for chemical and biological sensing [3]–[5]. However, the methods used to fabricate these sensors can be costly, complex, and incompatible with living tissues.

Polymers offer a favorable material for use in developing these tools for studying biological systems, due to their bio-

compatible nature, low cost, and flexibility of fabrication methods [6], [7]. Polymers can additionally act as sensitive layers, which is the prerequisite for the optimization of chemical and biosensors [8].

Deep UV (DUV) modification of polymethyl methacrylate (PMMA) can be used to create optical waveguides capable of guiding light in the visible optical spectrum (400–800 nm) [9]. Due to the optical clarity of PMMA in this region, fluorescent molecules can be selectively excited using this technology. Additionally, DUV modification modifies the surface chemistry of polymers affecting the selective adsorption of proteins and the adhesion of cells grown *in vitro* [10]. The bifunctionality of the modified polymer chips supporting both waveguiding and selective cell-anchoring capabilities can serve as a building block for future biophotonic ICs (Bio-PICs).

In this paper, we demonstrate that DUV-induced modification of PMMA can serve as the core technology for realizing optical-waveguide-based devices for controlled depth monitoring of cellular processes. The advantage of using this technology is that only one material is required, no etching or deposition is required, the materials used are biocompatible, and the number of steps is reduced when compared to other processes [11], [12]. By exploiting this technology devices can be designed that are precise, cheap, and easy to fabricate and use.

II. MATERIALS AND METHODS

Optical waveguides are extremely useful devices used for sending optical signals over very long distances with very little attenuation [13]. Although developed and used primarily for use in the telecommunications industry, waveguides have proven to be useful in other areas including in biological and chemical sensing [14], [15]. Optical waveguides operate on the principle of total internal reflection (TIR), whereby light in a core material is totally reflected at the interface with a substrate and cover material resulting in light being guided within only the core material. Snell's law can be used to show that light within the core material with refractive index n_w can be completely contained, as long as the substrate material has a refractive index $n_s < n_w$, the cover material has a refractive index $n_c \leq n_s < n_w$, and the angle of the light is less than a critical angle θ_c defined by

$$\theta_c = \arcsin\left(\frac{n_s}{n_w}\right) \quad (1)$$

as shown in Fig. 1.

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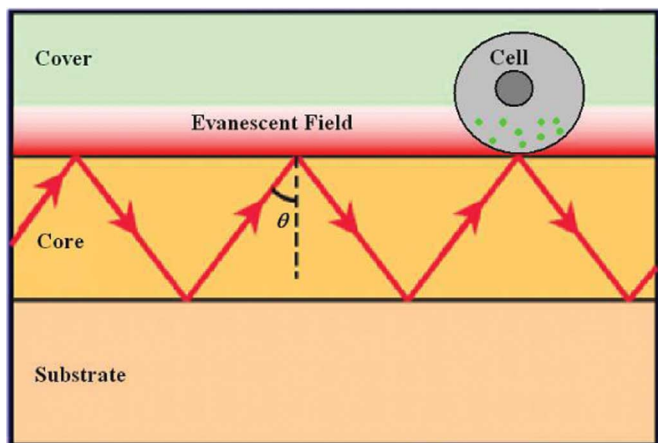


Fig. 1. Illustration of light being confined to the core of an optical waveguide. The evanescent field is generated at the interface between the core material and the cover material. A cell is illustrated on top of the waveguide to demonstrate how the evanescent field would interact with fluorescent molecules within it near the interface with the core material. It should be noted that the image is not to scale.

At the interface of the core material and cover material, an evanescent field is generated that extends some hundreds of nanometers into the cover medium where light is totally internally reflected, as shown in Fig. 1. This field is caused by the fact that electromagnetic fields cannot be discontinuous at a boundary, as would happen if there were no evanescent field. Evanescent fields are at the same wavelength as the light used to generate them. The intensity of evanescent fields decays exponentially as the distance from the interface is increased according to

$$I(z) = I_0 e^{-z/d} \quad (2)$$

where I_0 is the intensity of light at the interface, z is the distance from the interface, and d is the penetration depth of the evanescent field, defined as the distance from the interface where the amplitude of the electric field has decayed to $1/e$ of its original intensity, and given by

$$d = \frac{\lambda}{4\pi\sqrt{(n_1^2 \sin^2 \theta - n_2^2)}} \quad (3)$$

where $n_1 > n_2$, λ is the wavelength of the incident light in vacuum. These evanescent fields can be used to excite fluophores located within several hundred nanometers of the interface between the core and cover mediums. This technology has previously been employed in the TIR fluorescence (TIRF) microscope to monitor cell properties at the interface of the cellular membrane and growth substrate [16]. The TIRF microscope generally employs a complicated setup requiring prisms and only a very thin section in which cells can be grown [17], [18]. By using waveguides integrated into PMMA, cells can be cultured over long periods of time in a more conventional culture chamber and, due to its optical clarity, can be imaged from both above and below the culture chamber.

The polymer waveguides are fabricated using previously described methods [19]. Briefly, wafer blanks are fabricated

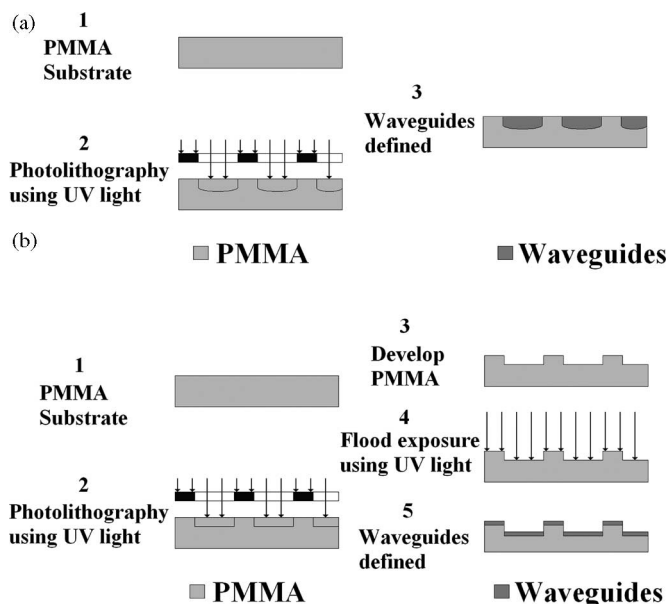


Fig. 2. Illustration of waveguide fabrication. The fabrication of planar waveguides, as shown in (a), is only a single step where the substrate is exposed to DUV light through a quartz/chromium mask resulting in the definition of waveguides. Ridge waveguide fabrication, as shown in (b), requires several steps including lithographic patterning through a quartz/chromium mask, development of the PMMA, and a subsequent flood exposure of the substrate, resulting in raised ridges with waveguides defined along the top.

using PMMA. The PMMA blanks are then patterned using a conventional photolithographic system incorporating a quartz/chromium mask. The mask aligner used is an EVG620 having a 100 W/cm mercury xenon arc lamp combined with a cold mirror with reflectance in the wavelength range of 200–240 nm in the exposure system. This system delivers 0.8 mW/cm² at 240 nm. The DUV light is absorbed by the PMMA substrate leading to cross-linking within a volume of substrate. The cross-linking results in an increase in the refractive index in the irradiated areas of the PMMA substrate and leads to the formation of planar waveguides by TIR, as illustrated in Fig. 2. Ridge waveguide are formed by developing the PMMA after patterning, which acts as a positive photoresist, resulting in ridges. The entire blank is subsequently flood exposed with DUV resulting in ridge waveguides, as illustrated in Fig. 2. Wafers are then diced using a polymer dicing saw, resulting in a facet at each waveguide capable of coupling light from an external fiber optic source. The DUV technique has several advantages with respect to common methods because only a single polymer layer is used, which serves as the substrate and waveguide as well and no further etching or development steps are required. The waveguides are fabricated at a dosage of 5 J/cm². PMMA is transparent in the wavelength range between 400 and 800 nm, which is ideal for biophotonic applications. The width of the used waveguides is between 5 and 20 μ m leading to multimode operation in this wavelength range.

PMMA has an increasing absorption peak below 200 nm, which is consistent with the generation and reaction of unsaturated bonds that were also determined by Raman spectroscopy [20]. This peak is due to a (PI)–(PI)* transition of C=C

bonds. Waveguide fabrication is done under vacuum conditions. In air, this absorption peak decreases for an irradiation dose higher than 2.16 J/cm^2 . This indicates the oxidative decomposition of the double bonds. At incident wavelengths above 220 nm, the wavelength region where photodegradation takes place, the optical penetration depth rapidly decreases with increasing irradiation dose to some few micrometers. This means that a few micrometer thin surface layer can be modified in a thicker PMMA bulk layer with a higher refractive index. Therefore, not only the refractive index increase determines the necessary irradiation dose, but also the existence of a certain threshold dose, which must be exceeded to guarantee a thin enough modified layer for waveguiding. With an increasing dose, both in air and in vacuum, the carbonyl band in the pendant group ($-\text{COOCH}_3$) of PMMA decreases and shifts toward higher absorption frequencies, indicating a change of the chemical environment. Furthermore the C–H stretching and bending absorption bands and the C–O stretching bands decrease substantially. This behavior is due to a removal of the ester side group followed by a main chain scission or the generation of unsaturated bonds in the polymer chain. The yield of double-bond formation in the main chain is larger than the main chain scission with UV irradiation.

Cell culture chambers are added to the PMMA substrates to maintain cell growth for several weeks. Chambers are custom machined out of polytetrafluoroethylene (PTFE) to an area of $\sim 500 \text{ mm}^2$ and a height of 10 mm. Chambers are then attached to the substrate using polydimethylsiloxane (PDMS), as shown in Fig. 3. The PDMS is degassed under vacuum and then cured at 58°C overnight.

Primary hippocampal cells from embryonic day 18 Sprague/Dawley rats are grown directly on polymer waveguide substrates using a combination of several methods to promote cell attachment and adhesion [21], [22]. Due to the delicate nature of the PMMA, substrates cannot be sterilized using an autoclave or alcohol, both of which will destroy the sample. In addition, UV sterilization is impossible as it may interfere with the waveguides. Instead, long-term, low-heat sterilization was performed (56°C for 12 h). This form of sterilization had no effect on the polymer waveguides function and adequately sterilized the substrates for use in aseptic cell culture. Substrates are then functionalized using a solution of extracellular matrix (ECM) gel (E1270, Sigma-Aldrich Corporation, St. Louis, MO) diluted in Dulbecco's Modified Eagle's Medium (11885084, Invitrogen Corporation, Carlsbad, CA) in a ratio of 1:100, with $10 \mu\text{g/mL}$ of polylysine (PL) (P7405, Sigma-Aldrich Corporation) [22]. The substrate is incubated at 37°C and $5\% \text{ CO}_2$ for 4 h with the ECM/PL solution. After functionalizing, substrates are rinsed and incubated for 1–3 days with B27/Neurobasal media + 0.5 mM glutamine. One pair of hippocampi is obtained from Brainbits (hp2, BrainBits LLC, Springfield, IL) and is shipped in hibernate, a proprietary media which maintains cell viability for several days under refrigeration [21]. The cells are then plated according to previously described protocols [21]. Briefly, cells are then dissociated by pipetting cells in and out of a Pasteur pipette (with the tip fire polished to roughly half its original size) ten times or until most of the tissue is broken up. The cells are then centrifuged for 1 min at $200\times G$ and the supernatant is

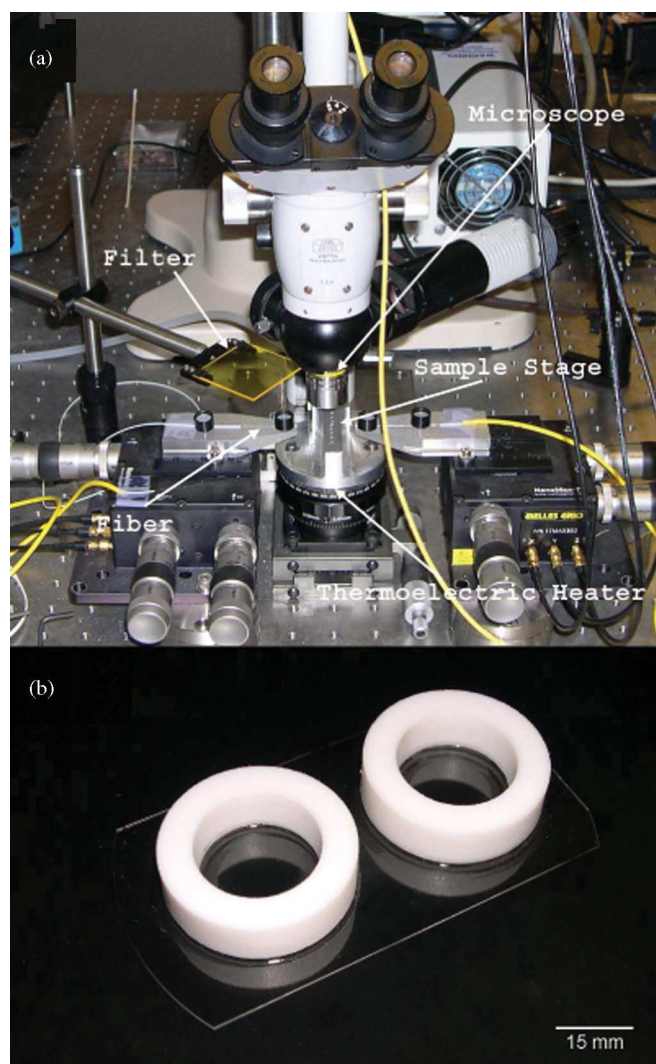


Fig. 3. Image of the experiment setup for optical recording. (a) Upright microscope, sample stage, coupling fibers, and low-pass optical filter. The 473 nm laser used to generate evanescent fields in waveguides is located to the left of the picture and is coupled in via the fiber on the left-hand side of the picture. The fiber on the right-hand side can be used to couple light out of the waveguides for further analysis. (b) Cell culture chamber attached to the substrate with integrated optical waveguides.

removed. Cells are then dispersed in 1 mL of B27/Neurobasal media + 0.5 mM glutamine + $25 \mu\text{M}$ glutamate. Cells are then plated at a density of 160 cells/mm^2 onto polymer substrates. A polystyrene lid is then placed over the culture chamber to maintain sterility and reduce evaporation. Cultures are maintained in an incubator at 37°C and $5\% \text{ CO}_2$. One half of the media is replaced every 3–4 days with fresh, prewarmed B27/Neurobasal + 0.5 mM glutamine.

At 4–14, days cells are loaded with Fluo-4 (F-14217, Invitrogen Corporation), a calcium-sensitive dye, using previously described methods [23]. Briefly, cells are incubated in prewarmed Ringer's solution [in sterile Hank's buffered salt solution (HBSS) 2.5 mM CaCl_2 , 1.3 mM MgCl_2 , 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH adjusted to 7.4] with 10 mM Fluo-4 for 30 min in the dark at room temperature. Cells are then incubated for another 30 min in

indicator-free Ringer's solution at 37 °C and 5% CO₂. Ringer's solution is designed to maintain healthy cell cultures outside of an incubator for a short period of time. Experiments are carried out within 1 h of the cells being loaded with Fluo-4.

Cell cultures are mounted to a custom designed monitoring system. Culture chambers are held down using a custom fabricated vacuum chuck. Attached to the chuck is a Peltier heater used to maintain the cell culture at 37 °C. Fiber optics can then be brought in from either side using a conventional XYZ stage, enabling excitation light to be inserted into the waveguides as well as be extracted. Coupling losses from fiber to chip can be as low as 0.5 dB/facet. A Zeiss upright microscope with long working distance objectives is used to align optical fibers to the waveguide facets as well as monitor fluorescent activity of cells on the waveguides, as shown in Fig. 3. Excitation light is provided by a diode-pumped solid-state (DPSS) laser operating at 473 nm, 100 mW output, and coupled using a graded-index (GRIN) lens (CFC-11-A-APC, Thorlabs, Inc., Newton, NJ). Fluo-4 has an excitation peak at 493 nm and an emission peak at 516 nm. At 473 nm, Fluo-4 is excited at 43% when compared to its maximum excitation wavelength [24]. A low-pass optical filter with cutoff frequency of 495 nm can be moved in and out the line of sight of the microscope to remove excitation light and transmit only emitted light.

III. RESULTS

Evanescent waveguide extension was confirmed to be confined to within several hundred nanometers of the substrate surface using near-field microscopy. This corresponds well with simulations, as seen in Fig. 4. Waveguides are tested using polystyrene fluorescent microspheres of various diameters. Microspheres with diameters of 10, 4, and 0.1 μm are used. Microspheres are maintained in a solution of deionized water with 0.15 M NaCl, 0.05% Tween 20, and 0.02% thimerosal with 3.6×10^6 particles/mL for 10 μm spheres, and deionized water with 2 mM sodium azide and 1.4×10^7 particles/mL for 4 μm spheres and 1.8×10^{11} particles/mL for 0.1 μm spheres (F-8836, T-72844, Invitrogen Corporation). All sizes of microspheres have an absorption peak at 505 nm and an emission peak at 515 nm, which matches quite closely to the fluorescent dye used in the cells. A 0.1 mL droplet of microspheres is pipetted onto the surface of the waveguides and visualized using a traditional epifluorescence microscope (Olympus IMT-2). After 30 s, microspheres have settled out of the solution and onto the surface of the PMMA. Fluorescent activity is verified first with the fluorescent microscope. Upon verification of fluorescent excitability, the sample is moved to the optical setup and excitation light is coupled into a single waveguide. All sizes of microsphere show good fluorescent excitation directly above optical waveguides, with significantly reduced excitation from beads not directly above waveguides. The 10 μm spheres are particularly bright and selective excitation can be seen without filtering the excitation light out. An increase of up to 4 dB is seen in the fluorescent intensity of spheres on waveguides compared to spheres off waveguides.

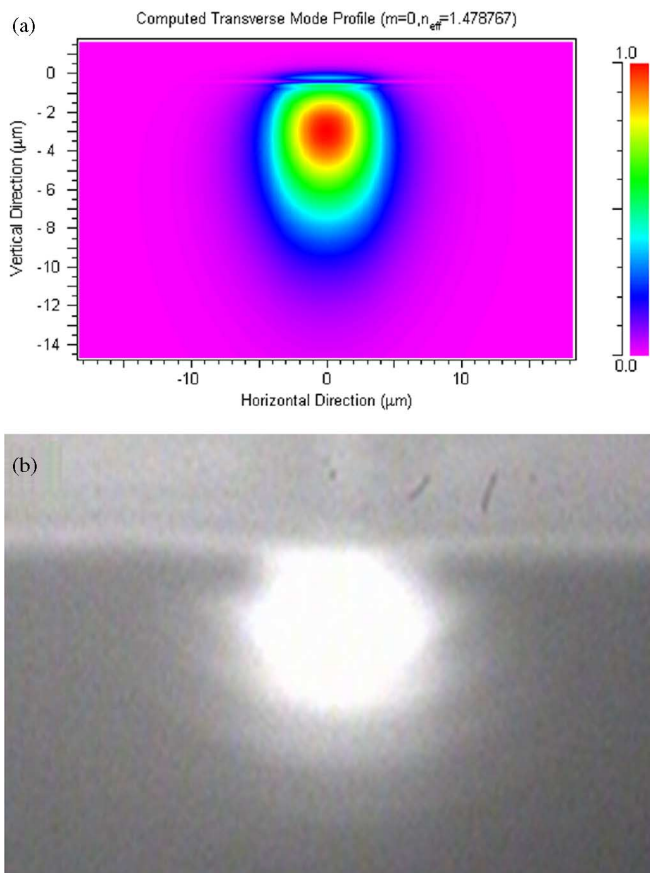


Fig. 4. Images of the evanescent field above optical waveguides. (a) Results of a simulation clearly showing the evanescent field being guided within a few hundred nanometers above the surface of the waveguide at $y = 0$. The majority of the power is carried within the core of the waveguide from $y = 0$ to $y = -11$. (b) Near-field micrograph of a waveguide. The evanescent field can be seen to extend a few hundred nanometers above the surface of the waveguide, visualized as the bright white spot in the center.

Cells are seen to attach well to the substrate, both on and off waveguides, are well spread and maintain good morphology for multiple weeks in culture. This indicates good compatibility with all materials used, as expected. Cells loaded with Fluo-4 exhibit good fluorescent excitability using a traditional epifluorescence microscope (Olympus IMT-2).

Typically, only one waveguide is illuminated at a time unless a y -splitter is incorporated into the design, in which case two waveguides can be monitored simultaneously. Substrates are initially visualized using normal illumination to find cells directly on waveguides. Once a suitable area has been found normal illumination is turned off, and the DPSS laser is turned on. Upon confirming that waveguides are well aligned by visual inspection, the low-pass filter is moved into place to eliminate visualization of excitation light. Images are then captured using a 3 megapixel Nikon Coolpix 4300 camera with custom microscope adapter (LNS-23D, Zarf Enterprises, Spokane, WA) using a 4 s exposure in a dark room. Images obtained are then analyzed in ImageJ to determine cell location and fluorescent excitation.

Fig. 5 shows cells grown directly on waveguides. The upper image shows cells grown on planar waveguides. It is observed

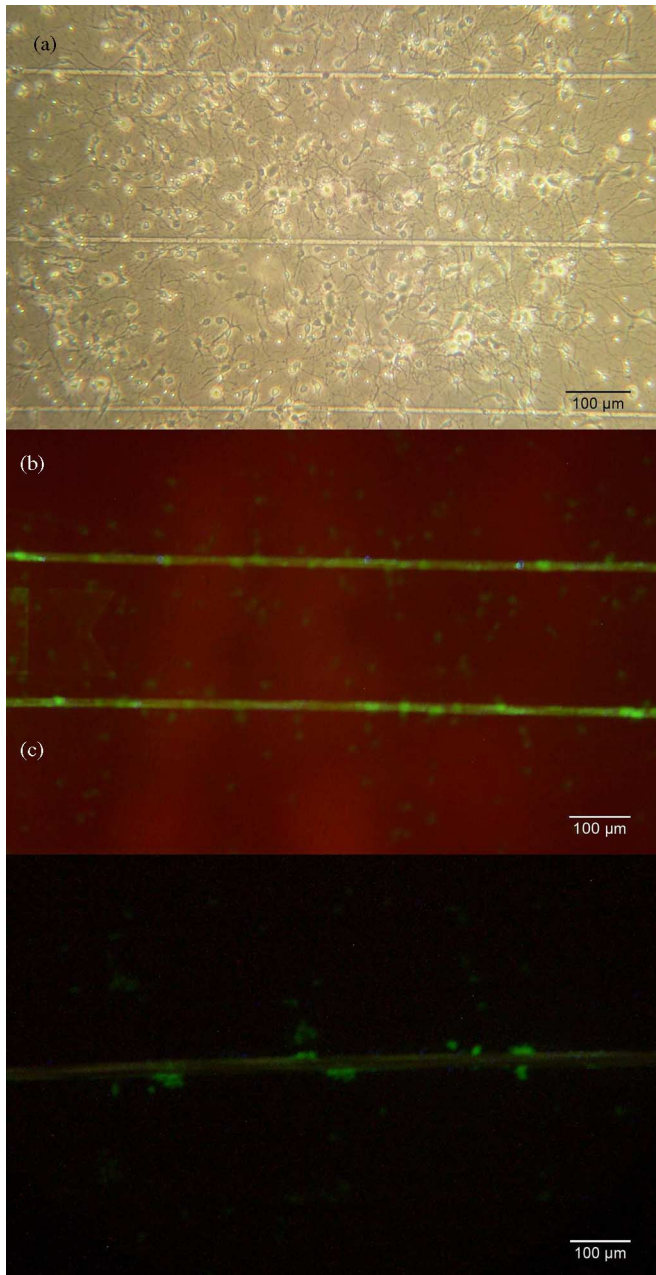


Fig. 5. Micrographs of neural cells being grown and fluorescently excited by the evanescent field produced by integrated optical waveguides. Neural cells grown directly on the polymer substrate, as shown in (a), show good attachment, cell growth, and morphology. Neural cells directly above planar waveguides, as shown in (b), are fluorescently excited when compared to cells not directly above planar waveguides. Neural cells directly above and next to ridge waveguides, as shown in (c), are fluorescently excited when compared to cells not in direct contact with ridge waveguides. It should be noted that cells tended to grow along the sides of the ridge waveguides as opposed to top, yet fluorescent excitation is still clearly visible compared to cells at some distance from the waveguide.

that two waveguides are illuminated as a result of a y -splitter not shown in the image. The lower image shows cells grown on ridge waveguides. It is noted that cells tended to grow on the sides of the ridges, as opposed to the tops. However, this does not affect the mechanism of fluorophore excitation by evanescent excitation. About 4 dB increase in fluorescent intensity was observed for both planar- and ridge-type waveguides.

Based on a refractive index of the PMMA waveguides of 1.495 and a refractive index for cells of roughly 1.38, the evanescent field is calculated to be between 100 and 200 nm deep into the cell, as shown in Fig. 5 [20], [25]. This is deep enough to penetrate the cell membrane to excite fluorophores within the cell, while simultaneously being shallow enough to offer an image of the basal membrane of the cell without much interference from fluorophores within the rest of the cell.

IV. DISCUSSION

Cells that grow in direct contact with the integrated waveguides are clearly seen to be fluorescently excited when compared to cells not in contact with the integrated waveguides. This indicates that cells are being fluorescently excited using the evanescent field associated with the waveguides. Although there is some small amount of fluorescent excitation observed in cells not located directly in contact with waveguides, it is at a much lower level than cells in contact with waveguides. This parasitic fluorescence can be explained by stray excitation light that is scattered at the interface where light is injected into the waveguide from the optical fiber. This is supported by the observation that light is at a higher intensity closer to the waveguide input as compared to further away. For this reason, there is a need to improve the waveguide–fiber coupling. In this way, cells can be selectively visualized by choosing the specific waveguide where the cell of interest resides. In addition, by using cell patterning techniques cells can provide even more sensitive results by placing cells in an exact relation to one another and to the waveguides themselves. It was noted that cells grown on ridge waveguides tended to grow on the large areas between waveguides and along the sides of the waveguides, but not on the tops of the waveguides. It was unclear if this was due to preferential adsorption of proteins onto etched portions of the substrate, or due to topographical cues from the ridge waveguides being raised several micrometers above the surrounding substrate [26]–[28]. In either case, extra care may need to be taken to ensure good contact of cells to the ridge waveguides.

Despite the presence of a low-pass filter used to block the excitation wavelength, the waveguides are still clearly visible when illuminated with the excitation light, as shown in Fig. 5. This has been established as being a result of fluorescent dye selectively adsorbing to DUV irradiated surfaces of PMMA substrates. It has been demonstrated that this background fluorescence can be quenched using fluorescein-specific antibodies (A889, Invitrogen Corporation). A decrease in fluorescence of up to -4.3 dB was observed with the addition of $25 \mu\text{g}$ of antibody per milliliter. Additionally, investigating other fluorescent dyes may identify other useful fluorescent biomarkers that selectively adsorb to unirradiated regions of PMMA substrates or do not adsorb to PMMA substrates, thereby reducing waveguide fluorescence without the use of quenching molecules. Competitive binding of multiple molecules at the substrate surface may lead to relevant fluorescent markers not being as strongly adsorbed, leading to a decrease in waveguide fluorescence at the wavelength of interest. This technique was illustrated by using $4.0 \mu\text{m}$ polystyrene beads labeled with four fluorescent

dyes with peak emission profiles at 430, 515, 580, and 680 nm (T-7283, Invitrogen Corporation) on waveguides to which Fluo-4 had been previously adsorbed. The SNR was seen to be significantly higher when monitoring emission in 580 and 680 nm wavelengths. Diminished SNRs, similar to cells loaded with Fluo-4, were seen at the 430 and 515 nm wavelengths.

Although cells are clearly visible as being illuminated using integrated waveguide excitation, the images are of relatively low resolution. This is due to the use of very long working distance (>10 mm) microscope objectives used in the upright microscope. However, this technology can be easily used with an inverted microscope with short working distance, high-magnification objectives, as is more customary in biological studies. This will provide high-resolution images of fluorescently labeled cells on waveguides. When combined with previously described improvements, this setup should produce results similar in quality to the TIRF microscope, with much less setup required as all that is required to use the system is to align a laser to the facet of the waveguide and insert a filter in the optical path to block excitation light and does not require complicated prisms and oil immersion to obtain results [29]. In addition, cells may be monitored using this setup and then returned to a growth chamber and monitored again at a later date.

Another promising direction for the technology is evanescent-field extension. By adjusting the thickness of the waveguides and the refractive index of the substrate material to be less than that of the cover material, a reverse-symmetry waveguide can be fabricated leading to an extension of the evanescent field to a micrometer or more. Horvath *et al.* have demonstrated a similar technology to monitor cell attachment to a glass slide on nanoporous silicon [30]–[32]. Rabus *et al.* have demonstrated evanescent-field extension using a thin film of PMMA on top of nanoporous silicon with a refractive index of 1.2 [33]. Integration of this technology could lead to whole cell studies, and, with refinement, may eventually enable optical sectioning of cells at a fraction the cost of a confocal microscopy system by subtracting previous images from new ones using software as the field is extended. If the depth of the evanescent field can be controlled by the user, then by acquiring images at different values of evanescent-field extension, successive images can be subtracted from one another, resulting in effective, optical section image stacks. A system was presented for selective fluorescent excitation of cells using the evanescent field associated with integrated optical waveguides. It was shown that fluorescence could be selectively excited in cells using optical waveguides. This serves as a proof-of-concept for a new system that can be used for probing biological samples to a specified depth. Although further optimizations need to be introduced to the system, preliminary results are very promising and warrant further study of this unique technology for use with biological systems.

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