SILICA COLLOIDAL CRYSTALS AS POROUS SUBSTRATES FOR TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPY

By

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A Thesis Submitted to the Faculty of the DEPARTMENT OF CHEMISTRY In Partial Fullfillment of the Requirements For the Degree of MASTER OF SCIENCE In the Graduate College THE UNIVERSITY OF ARIZONA

2005
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ACKNOWLEDGEMENTS

I would like to acknowledge all the people who have made a difference in my life, from my family and friends to all the teachers to mentors I have encountered over the years. I owe a special thank you to Dr. Mary Wirth, for her guidance, wisdom, patience, support and belief in me. I would also like to thank the Wirth group for their help and companionship; especially Dr. Takahira Tokimoto and Dr. Scott Cowell for their training which directly affected this thesis. I would also like to thank my committee members, Dr. Indraneel Ghosh and Dr. Jeanne Pemberton.

Of course, I would be nowhere without my family. I would especially like to thank my parents, Rand and Chris Bethea for all their support and love they have given me everyday of my life. Also, many thanks are owed to my little sister, Tancia, who has always been there for me in a way only a sister could be. You three have been my rock, a constant home no matter where we have lived. I would also like to thank my fiancé, Luis Velarde, for his love and constant encouragement. ¡Gracias por todo, mi amor! Lastly, and definitely mostly, I would like to thank God, for I have been blessed in so many ways and I owe everything in my life to the grace of the Lord.
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ABSTRACT

In cell biology and chemistry, total internal reflection microscopy (TIRFM) has proven to be a useful technique that allows the probing of cellular processes with high-signal-to-noise ratio imaging. However, samples on solid substrates limit the accessibility to probe processes on extracellular membrane surface closest to the microscope objective. Colloidal crystals provide a porous alternative to the traditional solid substrates. Thin crystals exhibit optical properties similar to that of a fused silica coverslip allowing for TIRFM in the same manner as with a typical coverslip as demonstrated by the observance of Chinese hamster ovary cells with fluorescently labeled receptors on both types of substrates. Accessibility of the cell membrane closest to the substrate and the ability to probe fluorophore orientation information was observed by the binding of TIPP-cy5 to the human delta opioid receptor.
Chapter 1
INTRODUCTION

1.1 Total Internal Reflection Fluorescence Microscopy

Total internal reflection microscopy (TIRFM) has proven to be a useful tool in the study and increased understanding of cellular processes and interactions. TIRFM, like other fluorescence microscopy techniques makes use of fluorophores to observe some process of interest, such as enzymatic reactions,$^1$ protein-protein interactions,$^2$ receptor-ligand binding and ion-channel in bilayers,$^3, 4$ cell mobility,$^5$ endocytosis and exocytosis,$^5, 6$ just to name a few. However, unlike traditional microscopy techniques, TIRFM makes use of evanescent wave properties to study fluorophores in a small section of a cell or aqueous solution near a substrate surface, improving the signal-to-noise ratio typically found in other microscopy techniques.

1.1.1 TIRF Physical Theory

When light travels through two media of different indices of refraction, the light can either be reflected or refracted at the interface. The behavior of the light as it travel from a higher refractive index material ($n_1$) to a second, lower refractive index material ($n_2$) is governed by Snell’s law (equation 1-1), where $\theta_1$ is the angle of incident light and $\theta_2$ is the angle of the refracted or reflected light. In typical TIRFM experiments for the study of cellular process, the first material is usually a glass ($n = 1.52$) or silica coverslip ($n = 1.46$) and the second is a lipid bilayer or cell membrane, with refractive indices between $n = 1.33$ and $n = 1.38$. 

\[ n_1 \sin \theta_1 = n_2 \sin \theta_2 \]  \hspace{1cm} (1-1)

Figure 1.1 is an illustration of light traveling through a material of higher refractive index into a material of lower refractive index. The incident light can either be refracted with perhaps some reflection (red line) or totally internally reflected (green line). Total internal reflection requires that the incident angle, \( \theta_1 \) be sufficiently high enough to result in \( \theta_2 > 90^\circ \). At the point where \( \theta_2 = 90^\circ \), \( \theta_1 \) is termed the critical angle, \( \theta_C \), and for all angles of \( \theta_1 > \theta_C \), light is totally internally reflected.

At this point of internal reflection an exponentially decaying electromagnetic field, or evanescent wave, with the same frequency as the incident light, is created in the second medium, as illustrated by the series of small parallel lines in Figure 1.1. The depth of this evanescent field is related to the wavelength and angle of incident light and the refractive indices of the two materials by equation 1-2.

\[
d = \frac{\lambda}{4\pi \sqrt{n_1^2 \sin^2 \theta - n_2^2}} \hspace{1cm} (1-2)
\]

Typical penetration depths for fluorescence microscopy range from tens of nanometers to a few hundred nanometers. This small penetration depth is what offers an advantage over other microscopy techniques that image an entire specimen. By only exciting a small region of the sample, within the first 200nm of the sample, the result is a higher to signal-to-noise ratio fluorescence image compared to that of other fluorescence microscopy techniques.
Figure 1.1  Incident light traveling from a high refractive index material ($n_1$) to a lower refractive index material ($n_2$) can be refracted (red line), at angle ($\theta_2$), with the possibility of some reflection (dotted red line). When the angle of incident light ($\theta_1$) is larger than the critical angle total internal reflection can occur (green line), producing an evanescent wave at the interface between the two materials.
1.1.2 TIRFM Instrumental Approaches

There are two basic instrumental approaches to TIRFM, the prism method and the objective method. Although there are several configurations for the prism method, only a general description will be given here. As illustrated in Figure 1.2a, a prism is used to couple the excitation light into the sample to create TIRF. Biological samples, which are usually aqueous, are placed in a liquid sample holder or on a coverslip and a quartz slide attached to a prism is placed over the sample opposite of the microscope objective. When the light is coupled through the prism at an angle sufficiently large enough for internal reflection at the water-quartz interface, an evanescent wave is produced in the aqueous sample. The positioning of the sample between the source of the evanescent wave and the objective is the main disadvantage of the prism method. This configuration limits not only access to the sample, but it also requires that imaging of fluorophores in the evanescent wave be done through the bulk of the sample.

The objective method, illustrated in Figure 1.2b, overcomes some of the shortcomings of the prism method. Using this method, the evanescent wave and imaging occur on the same side of the sample, allowing direct access to the sample. Specifically, incident light passes through the perimeter of a high numerical aperture objective which allows the light to approach large enough angles to achieve internal reflection. Typical objectives used have numerical apertures of NA=1.45, which allow for angles as high as 73º and allow the use of commercially available immersion oils between the coverslip and objective. For substrates and samples with refractive indices that are close in value, a higher incident angle is required for internal reflection. This higher angle requires the use of higher numerical aperture objectives, which require the use of special immersion oils.
and coverslips. While in general, the objective method does allow access to the sample, the major disadvantage is the limited access to the sample near the high refractive index solid substrate. For biological samples, specifically cells and lipid bilayers, on a solid substrate, diffusion of analytes under the sample may be inhibited by the attachment of the cell or bilayer to the substrate.
**Figure 1.2** TIRF method configurations. The prism method (a) consists of a quartz slide with a prism that is placed over the aqueous sample. Light is then focused through the prism to achieve internal reflection at the quartz slide-water interface. The objective lens method (b) consists of an objective through which light can be adjusted to achieve internal reflection at the coverslip-water interface through the objective.
1.2 Silica Colloidal Crystals

Highly ordered, face cubic centered (fcc), colloidal crystals of monodisperse colloids with controlled size can easily be deposited on a solid substrate and used for various applications. Here, the assembly of crystals, their properties, and applications will be reviewed briefly.

1.2.1 Colloidal Crystal Assembly

Researchers Stober, Fink and Bohn determined in the mid-1960s that silica colloid growth could be controlled in terms of size and monodispersity by the hydrolysis of alkylsilicates and condensation of silicic acid in alcohol solutions as shown in equations 1-3 and 1-4.8

\[
\begin{align*}
Si(OR)_4 + H_2O & \rightarrow (OR)_2Si(OH) + ROH \\
(RO)Si(OH) + H_2O & \rightarrow SiO_2 + 3ROH
\end{align*}
\]

Since then, further control over silica colloid formation has been achieved through careful study of reagent composition and concentrations.9

These newly improved silica particles have found many uses as colloidal arrays. In fact, efforts to form high quality crystals that can be used for various applications methods have been devised that result in virtually crack-free, durable colloid arrays. One such method is the pre-shrinking of colloids, achieved by heating at 300-600°C which drives water and ethanol (from colloid synthesis) from the particles and causes the formation of siloxane bonds.15 Pre-shrunk colloids can then be assembled into a crystalline array by several methods such as spin coating,16 vertical deposition,18 dip
coating\textsuperscript{19}, capillary force\textsuperscript{20}, and gravity sedimentation\textsuperscript{21}. Of these methods, the vertical deposition method, used in this study, although slow (from hours to days), produces ordered crystals of controllable thickness without the need for special equipment such as lithography.\textsuperscript{18}

Packed crystals may need to be modified to provide a more robust surface for applications that require surface modification. This may be done by sintering the array (heating at 1050\degree C), to fuse the colloids together, resulting in a mechanically robust material.\textsuperscript{22} However, as with calcination, sintering promotes the formation of siloxane bonds rendering the surface unreactive for chemical modification. In order to convert the siloxanes back to silanols, the silica surface can be rehydroxylated in an aqueous acid or base, leaving the surface available for chemical modification.\textsuperscript{23}

### 1.2.2 Colloidal Crystal Applications

One of the major drives in the past few decades to study silica colloidal crystals is their application as photonic band gap materials. This has sparked a major interest in improving the methods by which they are assembled in order to yield highly ordered materials. These periodic dielectric structures affect the propagation of light through the material resulting in Bragg diffraction based on the colloid diameter, number of layers and refractive index, giving the ability to tune the photonic band gap based on colloid size and crystal thickness.\textsuperscript{17, 18, 24, 25}

Other notable applications of these colloidal crystals are their use as substrates for supported lipid bilayers and as uniform chromatographic materials.\textsuperscript{12, 14} The ability to
change the curvature of the crystal depending on colloid diameter and the presence of water in the pores which provides a cushion for the membrane and allows the integration of integral membrane proteins make this material desirable for studies with lipid bilayers.\textsuperscript{14, 26, 27} On the other hand, the uniform and tunable pore sizes, high surface area, ability to withstand high electric fields and chemically modifiable surface makes them an ideal separation medium for proteins.\textsuperscript{12}
1.3 Focus of Research

Total internal reflection fluorescence has the advantage of a reduced signal-to-noise ratio in imaging cellular processes as compared to other fluorescence microscopy techniques. However, the need for a high refractive index material to achieve TIRF results in the use of a solid substrate that can potentially inhibit access to the sample at the sample-substrate interface. This is particularly detrimental to the study of interactions of proteins and/or peptides with the extracellular membrane surface by TIRF microscopy.

The major goal of this thesis is to explore the use of silica colloidal crystals as a sufficiently high refractive index material that allows both total internal reflection fluorescence and access to the sample at the sample-substrate interface.

In this work, as illustrated in Figure 1.3 cells cultured directly on the colloid surface are used to demonstrate the ability to achieve total internal reflection and probe accessibility to the sample surface. Specifically, Chinese hamster ovary cells which overexpress the human delta opioid receptor (HDOR) with and without an enhanced green fluorescent protein (EGFP) label will be cultured directly on the colloids and observed by TIRFM. The ability to diffuse peptide through the substrate so that it may bind to the receptors on the substrate side of the cell will be tested with a peptide ligand antagonist TIPP, labeled with a cy5 dye.
The goal of this research is to use silica colloidal crystals as a porous substrate through which total internal reflection fluorescence can be achieved while allowing access to the sample from the side of the substrate. Achievement of these goals is evaluated by the imaging of Chinese hamster cells grown on the colloidal crystal and probing binding of ligand to the human delta opioid receptor at the cell surface.
Chapter 2
EXPERIMENTAL METHODS

2.1 Assembly of Colloidal Crystal

Colloidal crystals were assembled from colloids made in-house that were heat treated for improved packing. Treated colloids were then packed by vertical deposition and sintered for stability, followed by rehydroxylation to provide a surface amenable to cell culture. The general colloidal crystal assembly scheme is illustrated in Figure 2.1.

2.1.1 Synthesis of Silica Colloids

Silica colloids of approximately 200 nm in diameter were synthesized using the Stober-Fink method. Specifically, to a 250 mL round bottom flask, 50 mL of 2M ammonium hydroxide in ethanol, 24 mL of 18MΩ H$_2$O were added and stirred at a slow rate. In a 150 mL beaker, 124 mL of filtered absolute ethanol and 7.2 mL of tetraethoxysilane (TEOS) was added. The ammonium hydroxide and ethanol were filtered using a polytetrafluoroethylene Nalgene filter (25 mm diameter, 0.2 µm pores). The solution from the beaker was then added to the contents of the round bottom flask slowly and allowed to stir for three hours at room temperature. To remove excess reagent from the newly formed colloids, the reaction solution was spun at 10,500 rpm for 10 min and the supernatant was discarded. The colloids were then rinsed several times by three cycles of sonication in ethanol, followed by centrifugation. Then, the colloids were rinsed with 18MΩ H$_2$O three times in the same manner as they were with ethanol. Cleaned particles were then allowed to dry at room temperature.
2.1.2 Formation of Colloidal Crystal

In order to prevent cracking in the final colloidal crystal, before packing colloids were placed in a pyrex beaker and calcinated, heated to 600ºC for 10 hours, three times. Between each calcination step, the particles were suspended in absolute ethanol and sonicated for at least 2 hours. After sonication, the ethanol was evaporated by passing compressed air through the suspension.

Triply calcined particles of approximately 170 nm were then assembled on silica coverslips by vertical deposition. To 20 mL of HPLC grade methanol, 0.1 g of silica colloid was added. The mixture was then sonicated for 2 hours, until the particles were fully suspended. The slurry was then diluted by adding 10 mL of slurry to 90 mL of methanol and sonicating for thirty minutes.

Silica coverslips were prepared for deposition by cleaning with boiling hot methanol and lens tissue, followed by UV-ozone treatment for 15 minutes. Clean coverslips were then placed in a small cylindrical 20 mL flask containing 10 mL of the diluted suspension. Flasks were then placed in an incubator at 50ºC for about 12 hours, until all methanol evaporated. As the methanol evaporates, the colloids self-assemble on the coverslip surface. One side of the coverslip was wiped clean using lens tissue and methanol. Crystals were then placed in an oven and sintered at 1050ºC for 12 hours. After the sintering process, which removes silanols on the silica surface, crystals were rehydroxylated in a tertbutylammonium hydroxide solution (pH 9.5) at 60ºC for 48 hours. Then, crystals were rinsed several times with deionized water followed by methanol and allowed to dry.
2.1.3 *Characterization of Silica Colloidal Crystals*

Crystals were characterized by spectrophotometry, atomic force microscopy (AFM) and scanning electron microscopy (SEM). UV-visible absorption measurements were performed using an Agilent 8453 spectrophotometer, using a silica coverslip as a blank. Atomic force microscopy was performed using a Digital Instruments Bioscope and SEM of platinum coated crystals was performed with a Hitachi S-4500 with Thermo-Noran digital imaging.
Figure 2.1 Colloidal crystal assembly scheme. Colloids are first synthesized by base-catalyzed hydrolysis of TEOS. Colloids are then washed and calcinated three times. Calcined silica is then suspended in methanol and assembled onto silica coverslips by vertical deposition. Self-assembled crystals are then sintered and rehydroxylated.
2.2 Cell Culture

Before cell culture silica crystals were cleaned using boiling methanol and UV-ozone treatment followed by autoclaving. Chinese hamster ovary cells that over-express the human delta opioid receptor without (CHO HDOR) and with (CHO HDOR-EGFP) EGFP were cultured by a cell culture facility in the Department of Pharmacology, in the University of Arizona. Cells were cultured directly on the sterilized crystal coverslips in Ham’s F12 with 10% fetal bovine serum, penicillin/streptomycin and 500 μg/mL antibiotics G418.
2.3 Fluorescence Microscopy

To observe whole cells on fused silica coverslips and colloidal crystals, substrates were removed from culture media, rinsed with BES-Tyrodes buffer,\textsuperscript{28} mounted and covered with buffer in a liquid sample holder designed for use with an inverted microscope (TE2000-U, Nikon). To excite EGFP, an argon ion laser (35-IMA-0840-015) provided the necessary 488 nm line. To excite cy5, a krypton ion laser (Ion Laser Technologies, ILT5470K) provided the necessary 649 nm line. Light from both lasers were combined by way of a dichroic mirror and passed through a polarizer, and electro-optic light modulator (EM200, Leysop) for polarization control. The laser beam transmitted from the modulator is then coupled into a fiber optic that directs the light to the back of the microscope to the objective (100X TIRF objective, NA 1.45, Nikon). Using the TIRF attachment, the laser beam can be adjusted to the edge of the objective at a sufficiently high angle to achieve internal reflection. Wavelengths for EGFP (excitation: 488 nm, emission: 508 nm) and Cy5 (excitation: 649 nm, emission: 670 nm) excitation and emission were selected by using filter cubes from Omega Optical. The EGFP filter set consisted of 485AF20 (XF1202), 500DRLP (XF2037), 510AF23 (XF3080) filters. An XF110-2 Alpha Vivid filter set consisting of 630AF50, 650DRLP, and 695AF55 filters were used for Cy5. The fluorescence from the excited fluorophores within the evanescent wave was collected by the objective and images were captured using an ICCD camera, Cascade II (512x512, Roper scientific). The TIRF experimental set-up and schematic is shown in Figure 2.2.
Figure 2.2  TIRF microscope schematic (a) and set up (b). Excitation wavelengths for EGFP and Cy5 were provided by way of a Kr and Ar ion laser. Polarization control was achieved by the use of an electro-optic modulator. Light from the modulator was coupled into a fiber optic that directed the light to the back of a microscope where light could be adjusted to the edge of the microscope objective to achieve TIRF.
3.1 Colloidal Crystals

The ability to achieve TIRF through the colloidal crystal depends on the crystal having similar optical properties as the fused silica slide on which they are packed and the ability to transmit light at the desired wavelengths. For this study, transmission of wavelengths in the visible range is necessary. The transmission of incident light through the colloidal crystal depends on crystal thickness and the size of the individual colloids. A colloidal crystal of less than 1\( \mu \)m in thickness was chosen in order to minimize the Bragg diffraction, which is prominent in thick crystals.\cite{18} For example, Figure 3.1 is a theoretical visible transmission spectrum of colloidal crystals of 1\( \mu \)m in thickness (blue line) and 10\( \mu \)m (red line) in thickness consisting of the same 200nm colloids. The spectra were calculated using the scalar wave approximation.\cite{29} The spectra show a strong Bragg peak for the thicker 10\( \mu \)m colloidal crystal which is greatly reduced with the thinner 1\( \mu \)m crystal.

For this study, crystals of 750 nm thickness, Figure 3.2a and 3.2b, were assembled by vertical deposition and then sintered and rehydroxylated. Sintering the colloids, heating at 1050\(^\circ\)C, causes the colloids to slightly anneal to each other, the result shown in figure 3.2b, a SEM of a sintered and rehydroxylated crystal cross-section created by
Figure 3.1  Theoretical absorbance spectrum of 1 μm (-) and 10 μm (-) thick colloidal crystals of 200 nm particles. The thicker crystal shows an increased absorbance at the Bragg wavelength compared to the thinner crystals.
Figure 3.2  SEM images of a colloidal crystal cross section after sintering and rehydroxylation. Scale bars correspond to 500 nm (a) and 200 nm (b). The divots and protrusions shown in (b) are indicative of the colloids attaching to each other during the sintering process.
breaking a colloidal crystal in half. The 170nm colloids exhibit divots and protrusions at locations where the colloids were attached to each other. These places where the colloids are joined together create a robust substrate that is not easily altered mechanically.\textsuperscript{22} The inability to easily disturb the crystal structure is important in this case so that cells may be easily cultured on the substrate without altering the crystal’s optical properties. Figure 3.3a and b are AFM and SEM images, respectively, of the colloid array. The AFM image is on the size scale of a typical Chinese hamster ovary cell used in this study. On this size scale, both in the AFM and the SEM the crystals are well-ordered with very few defects or cracks. The defects that do exist are small, generally less than a single colloid in thickness and a few colloids in length. These cracks are most likely due to the rapid vertical deposition and heating treatments. For the use of these arrays as substrates, imperfections can be tolerated only on the condition that the crystal maintains optical properties similar to those of fused silica in the desired wavelength range.

Figure 3.4 demonstrates the experimental differences in the UV-visible spectrum of crystals of different thicknesses. The two colloidal crystals of 4.5\,\mu m and 750 nm were assembled and treated in a similar manner. The spectrum of the 4.5\,\mu m thick colloidal crystal (red line) is adapted from reference 22, with permission. The spectrum of this thicker crystal exhibits a strong Bragg peak at 425 nm with a peak absorbance of 1.50. As in the theoretical spectrum, Figure 3.1, the thinner crystal (black line) exhibits a much broader and lower absorbance peak. The 750 nm thick crystal has a Bragg peak at 363 nm with a peak absorbance of 0.10. However, in the visible wavelength range, the highest
absorbance value is 0.05 at 404nm, allowing the transmission of most of the light between 400 and 700nm.
Figure 3.3  AFM (a) and SEM (b) images of a sintered, rehydroxylated colloidal crystal. Both images show that the colloidal crystals are generally well ordered with few defects.
Figure 3.4 UV-visible spectrum of a 4.5 μm colloidal crystal (−) adapted from reference 22 with permission and that of a 750 nm colloidal crystal (−). The 750nm colloidal crystal exhibits an increase in transmission over all wavelengths.
3.2 TIRF through Colloidal Crystals

As a first test of total internal reflection fluorescence using the colloidal array, TIRF using a silica coverslip was observed, and compared to that of a silica colloidal crystal. CHO-HDOR cells labeled with EGFP were cultured directly on the coverslips and arrays (Figure 3.5a and 3.5d, respectively). Cells were then transferred into BES-Tyrodes solution and imaged. EGFP was excited at 488 nm and emission centered at 510 nm was collected.

The over-expression of HDOR-EGFP in the Chinese hamster ovary cells as well as proteins that fluoresce at the same wavelengths as EGFP result in such intense fluorescence that by epi-illumination (incident light passed perpendicular to substrate) it is difficult to focus and distinguish between the apical and basal cell surfaces. This is demonstrated using both a fused silica coverslip and a colloidal array (Figure 3.5b and 3.5e). However, when the laser is adjusted to an angle above the critical angle (Figures 3.5c and 3.5f) only those fluorophores within the evanescent field are excited which is evidenced by the decrease in fluorescence in the images. This ability to achieve TIRF is due to the higher refractive index of the colloidal crystal as compared to that of the cell, in addition to the ability of the crystal to transmit light with little diffraction. For colloids as made, that is before calcination and sintering, the refractive index is low, \( n = 1.40 \). However, for calcined, sintered colloids, the refractive index is \( n = 1.46 \), which is the same as the refractive index of fused silica.\(^{22} \) Assuming that the colloids have a refractive index of 1.46, by equation 3-1 which takes into account the contribution
Figure 3.5  CHO-EGFP cells on a silica coverslip (a)-(c) and on a colloidal crystal (d)-(f). Images (a) and (d) are the white light images for the corresponding epi, (b) and (e), and TIRF (c) and (f) images. Images of cells on colloidal crystals exhibit similar epi and TIRF images as the fused silica coverslip.
of water in the pores, where \( n_{H2O} = 1.33 \), the effective refractive index of the colloidal crystal is \( n_{eff} = 1.43 \).

\[
\left(3-1\right)
\]

\[
\begin{align*}
    n_{eff}^2 &= 0.74 \cdot n_{silica}^2 + 0.26 \cdot n_{water}^2
\end{align*}
\]

For a CHO cell with a refractive index of 1.38, the angle needed to achieve TIRF from the colloidal crystal to the cell is 75°. However, the objective only allows a maximum angle of 73° as dictated by equation 3-2 in which NA is the numerical aperture of the objective, \( n \) is the refractive index of the objective optics, and \( \theta_{max} \) is the maximum angle allowed by the objective.

\[
\begin{align*}
    NA &= n \cdot \sin(\theta_{max})
\end{align*}
\]

Although such a high angle of 75° is not allowed by the objective, the presence of immersion oil and silica coverslip allow light to be coupled into the crystal at a sufficiently high enough angle to achieve TIRF at the crystal – cell surface as shown in figure 3.6 (red line). At the critical angle for light traveling from glass to the cell, 65°, light is refracted through the silica coverslip, at an angle of 71°, and then through the colloidal crystal at an angle of 75°, which results in TIRF at the cell membrane. For a typical glass coverslip with a refractive index matching that of the microscope objective, and a cell with \( n = 1.38 \), the range of TIRF angles allowed by the objective is from 65° to 73°. However, for the colloidal crystal assembled on a silica coverslip, the range of angles at the objective in which TIRF at the cell surface is possible is from 65° to 70°. At 70°, light is totally internally reflected at the coverslip – colloid interface, as shown by the green line in Figure 3.6. Although the range of angles is reduced by 3°, as depicted by the
gray triangle in Figure 3.6, the ability to achieve TIRF with the colloidal crystal is not noticeably more difficult.
Figure 3.6 Illustration of TIRF through the colloidal crystal as a porous support for a cell. Incident light from the objective/oil (or glass) is refracted through the silica coverslip and the colloidal crystal and then totally internally reflected at the cell surface (red line). When the incident light reaches greater than 70º it is totally internally reflected at the coverslip-crystal interface (green line). The range of angles lost due to the addition of the colloidal crystal is shown by the gray triangle.
3.4 Receptor Accessibility

To establish the availability of receptors to ligand, TIPP (a human delta opioid receptor antagonist) was labeled with a cy5 dye and added to CHO-HDOR cells (without EGFP) on colloids. Figure 3.7a is the white light image of cells for which ligand binding was observed. The blank image (Figure 3.7b), with no ligand, shows very little autofluorescence at the cy5 emission wavelengths. Since the binding constant for TIPP is $k_d = 0.5 \text{ nM}$, when 1 nM TIPP-cy5 is added, the majority of the available receptors should be bound to ligand. Figure 3.7c is the epi-fluorescence image of 1nM TIPP-cy5 added to the CHO-HDOR cells. Compared to the blank it shows a significant increase in fluorescence. However, since all cy5 bound to the cell is excited, it is not possible to determine if there is any ligand bound to the basal surface of the cell. The mode of fluorescence was then shifted to TIRF and the focus was adjusted to the bottom of the cell. As demonstrated in the Figure 3.7d, there is ligand bound to the basal surface of the cell, indicating that ligand can diffuse through the pores of the colloidal crystal and bind to available receptors.
**Figure 3.7** CHO-HDOR (no EGFP) cultured directly on a colloidal crystal white light image (a) and fluorescence image at the Cy5 emission wavelengths (b). 1 nM of TIPP-Cy5 was added to test receptor accessibility in epi (c) and TIRF (d) focused at the basal cell membrane.
3.5 Polarization Studies

3.5.1 TIRF Polarization Background

One of the major advantages of TIRF is the ability to discriminate differences in polarization of fluorophores within the evanescent field, not accessible by epi-fluorescence. Figures 3.8 and 3.9 illustrate the polarizations that can be achieved in each mode of illumination, and the coordinate system for TIRF, respectively. The circle with a dot in the middle indicates that the electric field vector is perpendicular to the plane of the paper. In epi-fluorescence, Figure 3.8a and 3.8b, the electric field polarization before and after passing through the substrate is the same. Since the electric field is perpendicular to the propagation of light, in epi-fluorescence, in which the light is perpendicular to the samples, the only polarizations that can be accessed are those parallel to the substrate. In TIRF however, Figure 3.8c and 3.8d, fluorophores polarized both perpendicular and parallel to the substrate can be observed. Although, Figure 3.8d does show that the evanescent field has a slight elliptical polarization, as the incident angle approaches the critical angle, the parallel component approaches zero, resulting in an evanescent field that is almost completely polarized perpendicular to the substrate. As demonstrated in Figure 3.9, s-polarization refers to polarization along the x-axis (parallel to substrate) and p refers to polarization along the z-axis (perpendicular), and they will be referred to by this notation in the remainder of the discussion.
Figure 3.8 Electric field polarization in epi illumination (a) and (b), and in TIRF illumination (c) and (d). ♦️ represents the electric field vector perpendicular to paper. ⏶️ represents elliptically polarized light consisting mostly of the perpendicular component.
Figure 3.9  TIRF coordinate system. S-polarization corresponds to the electric field vector oriented parallel to the x-axis and p-polarization corresponds to the electric field vector oriented parallel to the z-axis, perpendicular to the substrate.
3.5.2 Polarization of TIPP-Cy5

Using the same cells as in Figure 3.7, Figure 3.10a and 3.10b are TIRF images of 1 nM TIPP-Cy5 bound to the basal membrane of the CHO-HDOR cells. P-polarization is shown in Figure 3.10a and s-polarization in Figure 3.10b. Visually, the fluorescence from fluorophore excited in the s-polarization is greater than that of fluorophore excited in p-polarization. Since it is most likely that the receptors are randomly oriented in the plane of the substrate, changes in the polarization of the incident light in epi-fluorescence would yield fluorescence images with the same intensity and the p-polarization would not be accessible. However, here in Figure 3.10 there is a significant difference in fluorescence intensity, indicating that there are fewer fluorophores oriented along the z-axis as those oriented along the x-axis.
**Figure 3.10** CHO-HDOR cell on silica colloidal crystals in p-polarization (a) and in s-polarization (b). The difference in fluorescence intensity between the two images supports the ability to probe orientation information.
Chapter 4

CONCLUSIONS AND FUTURE WORK

4.1 Conclusions

This is the first time that silica colloidal crystals have been demonstrated as porous substrate through which TIRF microscopy can be achieved. It has been determined that colloidal crystals of less than 1μm thickness have optical properties which are nearly the same as those of a silica coverslip (Figure 3.2 and Figure 3.4b). The thin crystals showed significantly increased transmission over all visible wavelengths as compared to a thicker colloidal crystal, making the possibility for TIRF more realistic.

When CHO-HDOR-EGFP cell were cultured on a coverslip and on a crystal (Figure 3.3), both showed a significant drop in fluorescence when the illumination method was changed from epi to TIRF. To test membrane receptor accessibility at the substrate, TIPP-cy5 binding to CHO-HDOR cells was observed and by focusing on the basal membrane it was determined that ligand was binding to the cell. This indicated that the crystal indeed provides a means for analyte to gain access to the bottom of the sample. In addition, it was demonstrated that orientation of the TIPP-cy5 ligand molecules could be probed using this method. The difference in intensity between the p-polarized and s-polarized images (Figure 3.10) indicated that there was an excitation orientation not achieved by epi that could only be achieved in TIRF. Although there are still improvements to be made and applications to explore, this work has successfully established that silica colloidal crystals are porous substrates on which cells can be
cultured and total internal reflection fluorescence achieved while allowing access to the sample at the interface with the colloidal crystal.
4.2 Future Work

4.2.1 Exploration of New Materials

Although it was determined that TIRF could be successfully achieved through the 750 nm thick colloidal crystals used in this study, the use of alternative materials could even further improve this new method. For example, thinner colloidal crystals would result in a material more close in optical qualities to that of fused silica and allow greater transmission of light. However, the use of a higher refractive index crystal of similar or decreased thickness would result in the recovery of the range of angles lost by using the silica colloidal crystal in addition to a reduction in the penetration depth of the evanescent field (equation 1-2) . Although recovering the range of angles lost in use of a silica crystal is not a major advantage, the reduction in the evanescent field will greatly improve the signal-to-noise images obtained using this method. This higher refractive index material can be achieved through the use of zirconia colloids (n = 2.0) or core-shell composite colloids of silica@zirconia with a tunable refractive index.\textsuperscript{32, 33} To match these higher refractive index materials commercially available objectives with NA = 1.65 and n = 1.78 and the corresponding available immersion oils and coverslips can be used.

4.2.2 Application for Native Cell Membrane Fragments

As an alternative method to studying membrane proteins in reconstituted lipid bilayers, researchers have begun to look toward using native cell membrane fragments. This method is advantageous in that it does not require time intensive purification that may result in the inactivation of receptors. To the contrary, using membrane fragments
provides a much shorter sample preparation and since the receptors are in their native membrane they are most likely correctly folded and have the machinery in the membrane they need to function. Membrane fragments have been immobilized on substrates by several methods, most which result in the inaccessibility of one side of the membrane and sometimes unknown membrane orientation (with respect to the substrate). However, an innovative approach to suspend membrane fragments across nanoaperature arrays has been reported. However, this technique requires lithography to create nanopores in a silica substrate and the flow-through set up limits the possibility of TIRF imaging. Colloidal crystals, however, are easily assembled as thin optically transparent substrates, making them more ideal for studying proteins in membrane fragments by TIRF microscopy.

4.2.3 Preliminary Membrane Fragment Results

As a first test of applicability, membrane fragments were formed directly on the crystal by pressing another crystal on a quartz slide (modified with silane) into the subconfluent layer of CHO HDOR-EGFP cells. The crystal on quartz was prepared in the same manner as the crystal on which cells were grown and then modified using 3-aminopropylidimethylethoxysilane. The silane provides a positive charge that electrostatically interacts with the negatively charged cell membrane so that when the modified crystal is removed from the cells (as shown in Figure 4.1a), the upper membrane rips away from the cell leaving a cell membrane fragment. Figure 4.1b-d are images of a cell membrane fragment on the colloidal crystal. The epi (figure 4.1c) and
TIRF (Figure 4.1d) show major differences in fluorescence, which is most likely from labeled receptor or other fluorophores that are present on top of the membrane fragment. These fluorescent species were most likely unable to be washed away after cell fragmentation. This problem can most likely be addressed easily by inverting the crystal with the cell so that intracellular contents are pulled away from the membrane fragments by gravity. However, the fact that membrane fragments can be formed on the colloidal crystal and are easily imaged are promising for the possibility of silica colloidal crystals as porous supports for total internal reflection microscopy of membrane fragments.
Figure 4.1  Schematic of cell membrane fragment formation (a) and white light (b) epi-fluorescence (c), and TIRF (d) images of a cell membrane fragment. Intense fluorescence in the epi- image is most likely due to internalize receptors in organelles that were not washed away after cell membrane fragmentation.
REFERENCES


