Characterization of functionalized glass and indium tin oxide surfaces as substrates for super-resolution microscopy

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Abstract
Modern high-throughput biosensors with sensitivity down to a single analyte molecule may be possible with single-molecule localization microscopy (SMLM). Functionalized surfaces can be fabricated with self-assembly monolayer chemistry on indium tin oxide (ITO) substrates but not glass. However, characterizations of SMLM-compatible fluorophores are primarily performed on glass substrates. Here we collect single-molecule kinetics data of isolated Alexa Fluor 647 molecules on bare and functionalized glass and ITO surfaces. Extracting the photophysical dynamics of the fluorophores allows direct comparison of behavior of this dye on these substrates and fitting data to a model that accounts for multiple reversible dark states. All surfaces had sensitivity sufficient to image single fluorophore molecules. Photophysical kinetics observed are similar between the two substrates. The photon yield from individual fluorophores was greatest on bare glass, but functionalized ITO surfaces showed superior yield to functionalized glass surfaces and nearly matched the yield of bare glass. Together these results indicate functionalized ITO as a promising substrate for modern single-molecule biosensors.

Keywords: biosensor, single-molecule imaging, super-resolution microscopy, indium tin oxide

(Some figures may appear in colour only in the online journal)
substrate to allow high-numerical-aperture objectives to probe this surface while isolated from the analyte environment. Glass is the classic choice with a long history as a substrate for SMLM. However, modification of glass surface chemistry in a complete and controlled manner is a difficult task [6]. Indium tin oxide (ITO)-coated on glass overcomes these obstacles and presents a surface more amenable to precise modification to target desired analytes [7]. As a further advantage, the ITO surface is electrically conductive which provides additional routes for analyte read-out and dynamic surface modification desired in modern biosensors.

The use of ITO as a substrate for SMLM has been demonstrated in recent works. These have shown compatibility of this surface for modification supporting growth of various mammalian cells. Cell response to introduced drugs could be probed through light microscopy or in bulk electrical impedance measurements [8, 9].

Given the distinct nature of ITO at the surface chemistry level, a characterization of fluorophore dynamics pertinent to SMLM on ITO versus glass surfaces has not been performed. Given the conductivity characteristics of these two substrates we expect to observe differences in dye behavior, especially for dyes close (<15 nm) to the ITO surface [10].

Here we characterize the relevant photophysics of a popular fluorophore for dSTORM, Alexa Fluor 647, on bare and modified glass and ITO surfaces. To do so, we developed a mathematical model that accounts for two reversible dark states and fitted experimental data to extract rate constants and fractions. ITO and glass surfaces were modified with linkers of increasing length and the photophysics of Alexa Fluor 647 in dSTORM buffers extracted with a custom-written analysis. Photon counts, localization precision, and blinking dynamics of this dye on these surfaces is presented to demonstrate the relative effects of substrate on the ultimate readout capabilities of SMLM on devices built upon these surfaces.

Methods

Surface synthesis

ITO surfaces were then modified using the procedure outlined previously [7]. Octylphosphonic acid, dodecylphosphonic acid and octadecylphosphonic acid monolayers were adsorbed on clean ITO surfaces from 1 mM tetrahydrofuran (THF) solution for 24 h to make octylphosphonic acid (ITO-8C), dodecylphosphonic acid (ITO-12C) and octadecylphosphonic (ITO-18C) surfaces, respectively as shown in scheme 1. After that, the modified substrates were gently rinsed with THF and then annealed at 200 °C for 48 h under vacuum to promote stable covalent bonding formation. The annealed surfaces were then rinsed with copious amount of THF and methanol. Subsequently, the substrates were thoroughly rinsed with Milli-Q water and dried in vacuum chamber at room temperature.

After cleaning, the glass surfaces were immersed either in 0.2% (v/v) trichlororhexilislane in benzene or trimethoxyoctadecylsilane in toluene for 2h to make Glass-8C or Glass-18C surfaces, respectively [11]. After that, the modified substrates were rinsed with toluene, benzene, methanol and Milli-Q water and dried in vacuum chamber at room temperature. All the surfaces were prepared immediately prior to use.

A solution of Alexa Fluor 647 C2 maleimide (10 mM, 10 µl) was added to a solution of bovine serum albumin (BSA, 2.5 mg ml⁻¹, 625 µl) in PBS (pH 7.0). The reaction vessel was sealed and placed in the dark at 4 °C. The solution was pipetted into Zeba spin desalting column (7K MYCO) to remove the excess Alexa Fluor 647 C2 maleimide. The degree of labelling is 90% as determined by UV/Vis spectroscopy, which means 90% of BSA molecules in the conjugate are labelled with Alexa Fluor 647. Each BSA molecule has only one cysteine residue and can be functionalized by only one fluorophore

A solution of BSA-Alexa Fluor 647 was diluted to 1 nM in PBS (pH 7.0) A few drops of the solution (50 µl) were then pipetted onto clean glass or ITO coated glass coverslips with incubated for 20 min in dark conditions at room temperature. The incubated substrates were then rinsed with PBS (1 × 15 ml) and stored at 4 °C in the dark prior to imaging.

Single-molecule microscopy

A TILL Photonics iMic TIRF system was used for imaging Alexa Fluor 647 molecules on prepared surfaces. This microscope included a 640 nm diode laser for illumination, reflected
by a quad-band dichroic (Semrock Di01-R405/488/561/635-25 × 36) through a 100× Plan-apochromat 1.46 NA oil immersion objective (Zeiss 420792-9800-000) before reaching the sample. The illumination angle was controlled by a galvo mirror conjugate to the sample plane which moved the illumination spot between four points around the objective back focal plane during each acquisition frame. These points were calibrated using a built-in routine in the instrument software to yield critical illumination from four orthogonal angles at the sample with the aim of improving illumination homogeneity.

Fluorescence collected by the same objective passed through the dichroic, a quad-band emission filter (Semrock FF01-446/523/600/677-25), and onto an Andor iXon 897 EMCCD camera. Camera pixels corresponded to 91.9 nm at the sample.

Samples were imaged in a magnetic sample chamber (Chemlide) under oxygen-scavenging STORM buffer consisting of base buffer of 25 mM HEPES, 25 mM glucose, and 5% glycerol, which was supplemented with 0.05 mg ml\(^{-1}\) glucose oxidase and 0.025 mg ml\(^{-1}\) horseradish peroxidase, and 50 mM cysteamine immediately prior to imaging. Mixed solutions and components were held on ice. All reagents sourced from Sigma.

For each imaged region of interest the active fluorophores were driven into a dark state with high excitation laser power for 1 min (1.25 mW measured after objective). Samples were then immediately imaged for 2000 frames at 100 ms exposure time with reduced laser power (1.0 mW measured after objective). An EMCCD gain of 250 was applied during the acquisition. Collected image stacks were saved as raw bytes with an associated Open Microscopy Environment compatible metadata file.

**SMLM analysis**

Custom scripts written in MATLAB (Mathworks, Boston, Massachusetts, USA) performed the post-acquisition data analysis. Raw image data corrected to reflect intensities in units of photons passed to the Localizer package [12] for localization of spots in each image frame; input parameters were a point spread function width of 1.3 pixels and intensity filter of 25. The Thompson equation [13] yielded uncertainties for each detected point. All detected points in an image stack generated a map that was binarized, blurred by a Gaussian with width of 8 μm, and segmented using a watershed algorithm. Further analysis considered all points falling within each segmented region as from a single molecule.

Extraction of kinetics from single-molecule clusters began with generating an intensity versus time vector for each molecule. Those frames where a particle was detected within a given cluster resulted in a value equal to the intensity of the detected molecule for that frame; frames with no detection for that cluster yielded a ‘0’ for the intensity in that vector position. For a given vector, a dwell time of intensities above 0 yielded on times for that molecule. Similarly, dwell times of that trajectory with an intensity of 0 yielded off times and a count of consecutive frames with values of 0 yielded the number of blink events for that molecule; in this step periods before or after the first or last, respectively, non-zero value were ignored. Finally, a sum of all intensities in a trajectory yielded the total number of detected photons from a molecule. Trajectories with particles present in the first frame and those with a total number of detected photons less than 150 were discarded and the remainder carried forward.

The pooled on time, off time, and number of blinks values for all trajectories of a given surface type were binned into probability distribution functions. Fitting these distributions to models for two-state decays for on and off times or a single power function for the number of blinks \(\eta\) parameter [14] (scheme 2). Pooled values for localization precision and photons per detection event were fit to two-component mixtures of 1D Gaussians. Pooled of on times, off times, number of blinks, localization precisions, photons per detection event, and photons per trajectory also generated mean and standard error of the mean (SEM) statistics.

**Results**

Glass and ITO surfaces were functionalized with alkyl silane or alkyl phosphonic acid, respectively, with varying lengths of the linking alkyl chain. Linkers 8 or 18 carbons long (here referred to as 8C and 18C surfaces) were applied to glass in addition to testing bare glass surfaces with no functionalization molecules present. ITO surfaces with linkers 8, 12, and 18 carbons, in addition to bare ITO, were similarly tested and referred to as bare, 8C, 12C, and 18C surfaces below.

![Scheme 2](image-url)
BSA-Alexa Fluor 647 was absorbed onto these surfaces and imaged using dSTORM microscopy. Fluorophores on a surface were driven into a dark state by illuminating the sample with 1.25 mW of excitation laser for 1 min prior to imaging. The laser was reduced to 1.0 mW and images captured at 10 Hz for 200 s. Fluorophores were detected within these stacks using the Localizer [12] routine in MATLAB and the table of detection events exported to a file.

Watershed segmentation of these detected events identified regions each containing a single active fluorophore. The detection events falling within each region were segmented to yield trajectories each representing a single isolated, active fluorophore molecule. From these segmented trajectories the relevant photophysics were extracted.

Differences were immediately apparent between glass and ITO surfaces as well as changes with linker length (figure 1). Glass surfaces showed superior localization precision of detected fluorophores compared to ITO surfaces across all linker lengths. Across all surface modifications, the localization precision of a single detection event on glass was 68%–73% smaller than that of ITO, with functionalized glass show mean precisions of 16.48 nm, 17.91 nm, and 19.94 nm, for the bare, 8C, and 18C surfaces, respectively, with the same ITO surfaces showing localization precisions of 24.18 nm, 25.57 nm, and 27.2 nm (figure 1(a)). The 12C ITO surface fits within the same trend with molecules on that surface yielding a mean localization precision of 26.88 nm.

These same observations were borne out in the photon counts per molecule on these surfaces as well (figure 2). Functionalized glass surfaces show an average number of photons per detection event of 347, 235 and 202 photons/event for the bare, 8C, and 18C surfaces (figure 2(a)). On ITO the mean number of photons per detection event is 82, 63, 64, or 56 for the bare, 8C, 12C, or 18C surfaces.

Both the localization precision and photon/detection distributions are markedly bimodal on functionalized glass surfaces (figures 1(b) and 2(c)). Fitting the peaks in the localization...
precision distribution for functionalized glass surfaces to two Gaussians allowed us to separate these two populations (figure 2(b)). One curve was centered at 13.17 nm, 12.14 nm, and 12.95 nm with the second population centered at 18.23 nm, 18.39 nm, and 22.56 nm for the three linker lengths tested on glass surfaces. These distributions appeared at ratios of 1:0.32, 0.46:1, and 1:0.85. The same treatment of ITO surface data gives distributions centered at 23.84 nm, 24.98 nm, 25.44 nm, and 26.12 nm for the four linker lengths tested. The largest distributions in the ITO data appeared in ratios more than 1:0.01 relative to a second population.

Fluorophores tracked through the timeline of the imaging acquisition showed more blinking (on-off-on) activity on functionalized ITO surfaces than on functionalized glass surfaces (figure 3). The mean number of blinking events per molecule under our imaging conditions for ITO surfaces was 9, 14.6, 12.3, or 13.0 for the bare, 8C, 12C, and 18C linker lengths (figure 3(a)). The values for functionalized glass surfaces were 3.0, 4.4, and 5.5 blinks/molecule for the bare, 8C, and 18C linker lengths.

The distribution of blinks/molecule for individual emitters could be fit to a geometric distribution defined by a single parameter, \( \eta \) (figure 3(b)). This distribution fits well for the distributions showing lower number for blinks/molecule (corresponding to mean blinks/molecule > 10). In the other distributions, very few representatives were seen for low number of blinks/molecule. However, the trend for this parameter fitting the single-emitter distribution followed that of the mean values, namely the \( \eta \) value increasing with linker length and values for ITO exceeding that of those surfaces built on glass. ITO surfaces showed \( \eta = 0.87, 0.95, 0.93, \) and 0.94 for the bare, 8C, 12C, and 18C surfaces; glass surfaces show \( \eta = 0.70, 0.76, \) and 0.83 (figure 3(c)).

These same molecular trajectories fitted well to a kinetics model of two ‘on’ states and two reversible ‘off’ states, differing between each other in the timescales of transition to the ‘off’ or ‘on’ state, respectively (scheme 2). Overall, molecules on ITO surfaces spent more time in the reversible ‘off’ states during these transitions, with mean off times of 1.07 s, 1.53 s, 1.11 s, and 1.64 s for the surfaces of increasing linker length (figure 4(a)). Fluorophores on functionalized glass surfaces showed off times of 0.79 s, 0.42 s, and 0.99 s for the bare, 8C, and 18C linker length surfaces. The kinetic rate constants of these transitions were similar for the slower rate; with fluorophores on all surfaces falling within the range of \( 0.54 \times 10^{-2} \) s\(^{-1} \) to \( 1.25 \times 10^{-2} \) s\(^{-1} \) with no clear trend seen with linker length or substrate (figures 4(b) and (d)). In contrast, the faster of the two rate constants was faster on surfaces built on a glass substrate (0.11 s\(^{-1} \), 0.17 s\(^{-1} \), and 0.08 s\(^{-1} \)) versus those on ITO substrates (0.080 s\(^{-1} \), 0.058 s\(^{-1} \), 0.077 s\(^{-1} \), and 0.053 s\(^{-1} \)).
The distribution factor between the two states, $\alpha$, shows a clear preference for the faster transition state on functionalized glass surfaces ($\alpha = 8.2$, 10.4, and 4.0 for bare, 8C, and 18C linkers, respectively) than those surfaces built on ITO ($\alpha = 3.1$, 1.7, 3.6, and 1.7 for bare, 8C, 12C, and 18C linkers, respectively) (figure 4(c)). The on time shows a similar difference based on surface substrate. The molecules on functionalized glass surfaces spent a greater time in the on state before transitioning to an off state. Mean on-times for fluorophores on glass surfaces were 0.44 s, 0.46 s, and 0.23 s for the bare, 8C, and 18C surfaces, with those on ITO showing mean on times falling between 0.11 s and 0.13 s in the surfaces tested (figure 5(a)). The kinetic rate constants for the two components of the fitted model show little difference between fluorophores on the two substrates, with the fast transition occurring between $0.07 \text{s}^{-1}$ and $0.19 \text{s}^{-1}$ and the slow transition with a rate between $0.56 \times 10^{-3} \text{s}^{-1}$ and $3.9 \times 10^{-3} \text{s}^{-1}$ with no apparent trend in linker length (figures 4(d) and (e)). The preference for the fast or slow state, $\alpha$, shows a slight trend towards the fast rate with increasing linker length on both glass substrates and ITO, increasing from 5.5 to 9.6 on glass and 12.5 to 20.7 on ITO (figure 4(c)).

Together these parameters all affect the total number of fluorophores detected from a single fluorophore on a given surface across the life of the molecule during an acquisition (figure 5). Their values decreased for fluorophores on glass surfaces from 465 photons/molecule to 398 photons/molecule from bare glass to that with an 18C linker. On ITO, this value was nearly constant from 433 photons/molecule to 443 photons/molecule across the tested surface modifications, highlighting the suitability of these surfaces for SMLM.

### Discussion

The results presented here demonstrate the compatibility of ITO surfaces with standard SMLM techniques. Fluorophores on functionalized ITO surfaces are amenable to single molecule imaging with a typical commercial TIRF microscope with only minor modifications to the critical angle for illumination.

In general, the fluorophores on ITO surfaces behaved similarly to those on glass surfaces. Most measured properties showed a general trend towards greater influence attributable to the buffer chemistry with an increase in linker length. For example, fluorophores on both glass and ITO surfaces showed increasing number of blinking events with increasing linker length i.e. distance from the surface. A decrease in on-time was also seen for glass surfaces with increasing linker length. We attributed this trend to greater access of the active buffer agents and attenuated influence of the surface environment on the tethered fluorophores as the linker length increases.

Glass surfaces gave a greater mean localization precision across all linker lengths. This could largely be attributed to the small, but present extinction of visible light from ITO. The ITO also presented a higher refractive index at the surface-buffer interface.
interface. Reflection of emitted light at that surface and the rate of decay of the illumination intensity is expected to be greater for ITO than for glass. Together these small effects all point towards ITO being a slightly less optically efficient surface for imaging. However, overall these effects were small and do not greatly impede the use of functionalized ITO surfaces for SMLM.

A large part of the perceived superior performance of glass with respect to localization precision of individual detection events is attributable to the ‘bright’ fraction of molecules on glass surfaces. These could likely be molecules adhering directly to the glass surface and not tethered to the linker molecules. The localization precision and photons/molecule values for glass surfaces were bimodal. Those of ITO surfaces showed strong monomodal character. Each glass surface’s bimodal distribution consisted of one peak sharing significant character with the primary peak of the bare surface. The other peak in each glass surface’s distribution was closer to that seen on ITO surfaces, with the overlap between peaks on different surfaces increasing with linker length of the surface functionalization. The position of this second peak ultimately overlapped with those from the ITO surfaces with sufficient linker length (see figure 2(b), 18C surfaces on glass and ITO).

If these are fluorophores adhering to the glass surface directly rather than tethered to the linker molecules as desired, this could be illustrative of the difficulty in generating a consistent surface on glass substrates. The functionalization of glass could be incomplete and leave areas of the surface open for non-specific absorption of dye molecules. This would be the case for analyte molecules as well and would be undesired background in a biosensor application. These bright detection molecules could also be fluorescent sites on the substrate surface or within the functionalized layer. If so, these represent another source of undesired background that is difficult to control on glass substrates. These ‘bright’ detection events did not occur on ITO. That points to the greater ability of ITO to be completely functionalized and resist nonspecific background absorption versus glass substrates.

Even with these potentially spurious bright molecules detected on glass, fluorophores on the ITO surfaces emitted a greater number of photons per molecule than glass surfaces with any linker length beyond the bare surfaces (figure 6). This effect can be attributed to the much greater number of blinking events a fluorophore on an ITO surface will cycle through before bleaching. A fluorophore on an 18C-functionalized ITO surface will emit 11% more photons in its detected lifetime than one on a comparable glass surface.

Previous work has shown that dye molecules in close (<10 nm) proximity to an ITO surface show measurable quenching, with molecules less than 1 nm from an ITO surface being non-emissive [10]. However, the fluorophores in this study are not bare dye molecules but rather conjugated to a BSA molecule (~14 nm × 4 nm × 4 nm). This acts as a spacer and this extra distance from the quenching surface may be sufficient to allow for dye fluorescence. This conjugation may also allow additional the dye to adopt dipole orientations not conducive to efficient quenching by the surface. Given that

Figure 5. On times for Alexa Fluor 647 on functionalized glass and ITO surfaces. Mean values with error bars of SEM across trials shown in (a). On time distributions in (b) are fit to extract values $\alpha$ (c), $k_d$ (d), and $k_{\text{on}}$ (e). Values in (c)–(e) are fits from (b) with error bars of the 95% CI of the fit.
Figure 6. Photons collected per molecule event for Alexa Fluor 647 on functionalized glass and ITO surfaces. Mean values ((a), error bars SEM and within data markers) and distributions for photons per detection event on studied surfaces shown.

dye fluorescence is observed in the Bare ITO condition, or where BSA is the only spacer between the dye and potentially quenching surface, the quenching effect is clearly not at a strength prohibitive of observing dye fluorescence on fluorophores conjugated to a stereotypical bioanalyte molecule. A closer examination of the quenching effect of ITO may be warranted in future studies where the target molecules or spacers are smaller than those chosen here.

The increased number of blinks per molecule of fluorophores on ITO surfaces provides a distinct advantage in correctly isolating desired molecules over background or spurious detection events in an SMLM experiment. The total number of photons yielded from each fluorophore on ITO is an added advantage providing a route to increased achievable analyte density in a biosensor application where detection events are grouped into molecular trajectories.

Conclusion

We demonstrate the compatibility of functionalized ITO surfaces for SMLM using conventional fluorophores and instrumentation. These surfaces show similar photophysical performance to more commonly-used functionalized glass surfaces and these differences are quantified and reported. The ITO surfaces show distinct advantages in terms of the functionalization chemistry and in the ultimate performance of the fluorophores in an SMLM acquisition. Together these data point to ITO as an excellent choice of substrate on which to build engineered surfaces for biosensor applications with single-molecule sensitivity and high throughput capacity.

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