Supplementary Note 1

Formulas for SAIM analysis.

Formulas used for the analysis plugin are drawn from supplementary reference [1]. This reference contained a typographical error in the r^{TE} formula (**3**), which has been corrected here.

For incident light polarized perpendicular to the plane of incidence, the detected intensity variation at a given pixel in the image relates to the average height of the fluorophore and the angle of incidence as follows:

$$I = A\left(\left|1 + r^{TE} e^{i\phi(H)}\right|\right)^2 + B \quad \textbf{(1)}$$

where:
A – accounts for variation in detected intensity due to factors including mean excitation laser intensity, fluorophore density, efficiency of emitted photon detection, etc..
B – is an offset parameter that accounts for background fluorescence in the sample images.
H – is the position above the silicon oxide layer
φ(H) – the phase difference of the direct and reflected light at axial position H given by:

$$\phi(H) = \frac{4\pi}{\lambda}(n_b H cos\theta_b) \quad \textbf{(2)}$$

r^{TE} – the effective Fresnel coefficient obtained from the transfer matrix m^{TE} according to:

$$r^{TE} = \frac{(m_{11}^{TE} + m_{12}^{TE} p_0)p_2 + (m_{21}^{TE} - m_{22}^{TE} p_0)}{(m_{11}^{TE} + m_{12}^{TE} p_0)p_2 + (m_{21}^{TE} + M_{22}^{TE} p_0)} \quad \textbf{(3)}$$

where:

$$m_{11}^{TE} = cos(k_{0x} d_{ox} cos_{ox}) \quad \textbf{(4)}$$

$$m_{12}^{TE} = \frac{-i}{p_1} sin(k_{0x} d_{ox} cos\theta_{ox}) \quad \textbf{(5)}$$

$$m_{21}^{TE} = -ip_1 sin(k_{0x} d_{ox} cos\theta_{ox}) \quad \textbf{(6)}$$

$$m_{22}^{TE} = cos(k_{0x} d_{ox} cos\theta_{ox}) \quad \textbf{(7)}$$

$$p_0 = n_{Si} cos\theta_{Si}, p_1 = n_{ox} cos\theta_{ox}, p_2 = n_b cos\theta_b \quad \textbf{(8)}$$

$$k_i = \frac{2\pi n_i}{\lambda} \quad \textbf{(9)}$$

k_i – wavenumber in the given material
n_{Si} – refractive index of silicon
n_{ox} – refractive index of the oxide layer
n_b – refractive index of the sample
θ_{Si} – angle of incidence in the silicon
θ_{ox} – angle of incidence in the oxide layer
θb– angle of incidence in the sample

By rewriting the effective Fresnel coefficient as a complex number:

$$r^{TE} = c + id \quad \textbf{(10)}$$

Equation 1 can then be rewritten as:

$$I = A(1 + 2ccos\phi(H) - 2dsin\phi(H) + c^2 + d^2) + B \quad \textbf{(11)}$$

Not only can equation 11 be computed about 10 times faster than equation 1, it can also be used to derive partial derivatives needed for Levenberg-Marquard non-linear least square curve fitting, using

$$f = \frac{d}{dH}\phi(H) = \frac{4\pi n_b cos\theta_b}{\lambda} \quad \textbf{(12)}$$

Partial derivative of equation 11 for H:

$$\frac{d}{dH}(I) = -2Af\left(csin\phi(H) + dcos\phi(H)\right) \quad \textbf{(13)}$$

Supplementary Note 2

Additional information about calibration

The SAIM calibration device is based on an Arduino open-source electronics platform. The device uses two linear CCDs (AMS-TAOS USA Inc., TSL-1412S) positioned above the microscope objective lens to detect fluorescence emitted by an acrylic faceplate when illuminated by an excitation laser (**Fig. 1d**, left). Based on the known, fixed vertical displacement between these two detectors the angle of that excitation light can be determined (**Supplementary Protocol**). The device is sized to fit in a standard 96-well plate stage insert.

The calibration device is controlled by a μManager [5] plugin to execute SAIM calibration and data acquisition. This plugin consists of two parts. 1) The “Calibration” panel communicates with the calibration device and defines the relation between the refractive index corrected angle of excitation light and position of a motorized TIRF illuminator by fitting the data with a cubic polynomial and storing the function parameters. The plugin will store calibrations for multiple channel groups that can be accessed from the acquisition panel. 2) The “Acquisition” panel reads the calibration for a given channel group, takes a user provided range of angles and the number of steps for sampling this range, and then runs a series of acquisitions at these angles.

Supplementary Note 3

Additional information about analysis plugin

This SAIM analysis software is a FIJI [6] plugin consisting of three parts. "SAIM Plot" plots theoretical predictions for the intensity distribution as a function of distance from the surface of the oxide layer. "SAIM Inspect" and "SAIM Fit" are very similar; however, SAIM Inspect will act on the average values of the ROI (for instance, the pixel under the cursor) and executes only a single fit, whereas SAIM Fit will analyze all pixels of the image stack. SAIM Fit will fit each pixel in the input stack and output a stack with 4 images. The first one is the height map (in nm), the second image has the r-squared values (an indication of the goodness of fit with values between 0 and 1, the closer to 1, the better the fit), the third image shows the values for "A", a scaling parameter that accounts for variation in intensity, and the last image shows the values for "B", an offset parameter that accounts for background fluorescence. For samples thicker than one half the wavelength of excitation light, both the Fit and Inspect tools can be given multiple starting heights to prevent the fitting code from getting stuck in local minima.

This plugin uses the equations from the Paszek et al. paper [Supplementary reference 1] with a few extensions (**Supplementary Note 1**). The code uses an unbounded Levenberg-Marquard optimizer that minimizes the square of the difference between observed and predicted values. Parameters are restricted to physically relevant values (for instance, no negative heights are allowed). Execution speed depends on image content, though we routinely analyze every pixel in a dataset of 86 images at 1024 x 1024 pixels in 2.5 minutes on a MacBook Pro with a 2.7 GHz Intel Core i5 processor. In practice, masking images to fit only relevant pixels can accelerate processing dramatically.

Supplementary Note 4

Measurement of exact oxide thickness.

Samples for SAIM are prepared on commercially available silicon substrates with an oxide spacer. Any thickness of the oxide spacer can be used, but we found that spacers of ~1900 nm provided the optimal periodicity of the SAIM function in the range of angles that could be sampled using a 1.20 NA water immersion objective (**Supplementary Fig. 2a**). We measured the oxide thickness of SAIM substrates using ellipsometry (**Supplementary Fig. 2b**). To perform SAIM measurements without access to an ellipsometer, oxide spacer thickness also can be estimated using SAIM. We imaged a monolayer of surface-bound fluorescent dyes of defined thickness and fit theoretical predictions for different oxide heights to the data until the known height of the sample was derived. This method will also correct for differences in apparent oxide height for multiple laser lines. We used a DiO, DiI, and DiD triple labeled supported lipid bilayer (SLB) for this purpose, and fit the oxide to the known height of 6.4 nm for a phospholipid bilayer (**Supplementary Fig. 2b-c**)[Supplementary reference 10]. In our experiments the apparent oxide height varied by as much as 10-20 nm between experiments; therefore, we recommend performing this measurement on the day of each experiment to minimize variability in measurements due to changes in the microscope and/or calibrations (**Supplementary Fig. 2d**).

Supplementary Note 5

Comments on imaging complex samples

The SAIM theory predicts intensity as a function of angle of the excitation light for a point source with infinitesimal axial dimensions. In practice, fluorophores may be distributed over multiple z-positions within a diffraction-limited spot [Supplementary references 2-8], and the measured fluorescence signal is the sum of the individual contributions of each fluorophore. The resulting intensity versus excitation angle curve is not necessarily representative of the averaged z-heights.

Small (40-200nm) fluorescent nano-sized beads have regularly been used to validate SAIM measurements, using the assumption that the height in SAIM is the bead radius [3]. To address this assumption, we modeled the appearance of a 200nm sphere, stained throughout, in SAIM. The model divides the sphere in 9 parts, calculates the SAIM curve for each part, and averages the predicted intensity for the 9 parts at each angle, weighted by the fraction of the sphere's volume contained in each part (**Supplementary Fig. 3a**). This model shows that the bead's SAIM curve resembles the SAIM curve of a point source at the radius of the bead; however, the intensity maxima and minima are lower, especially at low angles. Fitting the resulting data with the SAIM Inspect tool resulted in a predicted height of 99.4 nm (r2 of 0.93), similar to the radius of the bead.

We measured fluorescent beads using both SAIM and negative stain electron microscopy. For SAIM, beads were adsorbed onto silicon substrates with an oxide spacer of ~1900 nm. Angle dependent changes in bead fluorescence intensity were fit to the optical model to obtain the axial position of the bead center. These measurements were repeated for beads with nominal 20- or 50-nm radii, and fluorescence excitation wavelengths of 488 or 561 nm (**Supplementary Fig. 3b**). Though the optical model predicts that fluorescent nanobeads should be reliably fit by the SAIM function, we found that SAIM heights were consistently around 10 nm larger than heights derived from EM measurement (**Supplementary Fig. 3c**). This offset is consistent with previously published results using SAIM to measure fluorescent bead heights [3]. For this reason, we advise using fluorescent monolayers instead of fluorescent beads for SAIM validation going forward.

For fluorescent beads the distribution of fluorophores is well defined and easily modeled. However, the distribution of fluorophores in a biological sample, like a fixed cell stained with fluorescent antibody is more difficult to predict. In cases of thicker samples, careful sample preparation to minimize background is key. SAIM is theoretically capable of measuring heights up to 1 μm or so using multiple starting z-heights for the fitting code, but obtaining reliable data with enough photons and minimal photobleaching can be challenging.