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Calibrating Evanescent-Wave Penetration Depths for Biological TIRF Microscopy

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3 **Calibrating evanescent-wave penetration depths for biological**

4 **TIRF microscopy**

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6 *Short title:* TIRF image quantification

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36 ¹⁾ MO was a Joseph Meyerhof invited professor with the Department of Biomolecular
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38 2018-19.

39

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42 ABSTRACT. Roughly half of a cell's proteins are located at or near the plasma membrane.
43 In this restricted space, the cell senses its environment, signals to its neighbors and ex-
44 changes cargo through exo- and endocytotic mechanisms. Ligands bind to receptors, ions
45 flow across channel pores, and transmitters and metabolites are transported against con-
46 centration gradients. Receptors, ion channels, pumps and transporters are the molecular
47 substrates of these biological processes and they constitute important targets for drug
48 discovery. Total internal reflection fluorescence (TIRF) microscopy suppresses background
49 from cell deeper layers and provides contrast for selectively imaging dynamic processes
50 near the basal membrane of live-cells. The optical sectioning of TIRF is based on the exci-
51 tation confinement of the evanescent wave generated at the glass/cell interface. How deep
52 the excitation light actually penetrates the sample is difficult to know, making the
53 quantitative interpretation of TIRF data problematic. Nevertheless, many applications like
54 super-resolution microscopy, co-localization, FRET, near-membrane fluorescence recovery
55 after photobleaching, uncaging or photo-activation/switching, as well as single-particle
56 tracking require the quantitative interpretation of EW-excited images. Here, we review
57 existing techniques for characterizing evanescent fields and we provide a roadmap for
58 comparing TIRF data across images, experiments, and laboratories.

59 (193 words)

60
61
62 KEYWORDS: nanometer, near-field, fluorescence, excitation confinement, optical
63 sectioning, near-membrane.

64
65
66

67 **Quantifying total internal reflection fluorescence**

68 The purpose of TIRF is to selectively illuminate fluorophores that are right near a surface
69 and not illuminate fluorophores that are further into the volume, above the surface. Major
70 uses of TIRF include single-molecule detection (SMD) – e.g., for the localization-based
71 super-resolution microscopies – as well as studies of cell-substrate contact region of living
72 cells grown in culture. Typical biological applications are the investigation of cell adhesion
73 sites, of the dynamics of membrane receptors, single-vesicle exo- and endocytosis or
74 ER/plasma-membrane contact sites. In TIRF, the confinement of excitation light to a thin,
75 near-inter-face layer results in background reduction and contrast enhancement.

76 Many TIRF applications require knowing the depth of the illuminated layer, e.g., for
77 quantifying the motion of molecules or cellular organelles near the substrate. In principle,
78 that depth can be calculated from the local refractive index (RI) of the medium (n_1) and the
79 incidence angle of the illumination (θ) at a known wavelength (λ). But in practice, the actual
80 depth is much less certain. The incident light is often not perfectly collimated but in a range
81 of angles, which sets up a continuous range of penetration depths. Also, the optics used to
82 guide the incident beam upon the sample itself scatters light, some of which enters the
83 sample as propagating light, which does not decay exponentially with distance from the TIR
84 surface. Another source of uncertainty in TIR depth is that the sample's refractive index is
85 not known precisely, and it may contain non-homogeneities, producing scattering.

86 In this perspective article, we briefly recall the fundamentals of evanescent waves
87 (EWs) prior to discussing the concepts, methods and difficulties of calibrating TIRF in-
88 tensities. We suggest a protocol for TIRF image quantification to better control, compare
89 and share results. Other aspects of TIRF and TIRF microscopy have been covered in a
90 number of excellent reviews and tutorials [1; 2; 3; 4; 5; 6; 7; 8].

91

92 **How to ‘see’ EWs?**

93 Light impinging at an interface between two media^a having, respectively, RIs n_1 and n_3 with
94 $n_3 > n_1$ is partially reflected and partially transmitted. Snell's and Fresnel's laws govern,
95 respectively, the angles and intensities of the reflected and refracted beams. A discontinuity
96 is observed at a very oblique angle, above the critical angle $\theta_c = \text{asin}(n_1/n_3)$ at which the
97 reflected intensity becomes equivalent to that of the incident beam. Yet, there is light in the
98 rarer medium (n_1), because energy and momentum conservation at the boundary prescribe

^a an intermediate layer having a RI n_2 and thickness d_2 is neglected.

99 the existence of a near field, skimming the surface. This evanescent (vanishing) wave
 100 propagates on the surface (as does the refracted beam at the critical angle θ_c) but its
 101 intensity is decaying exponentially perpendicular to the surface. Typical decay lengths are a
 102 fraction of the wavelength and can be as small as the $\lambda/5$, depending on the angle of
 103 incidence, θ , **Fig. 1A**.

104 EWs are non-visible in the far field, but scattering or absorption couple out energy
 105 from the near field, measurable either as an intensity loss in the reflected beam ('attenuated'
 106 or 'frustrated' TIR), or as fluorescence excitation in a thin boundary layer above the
 107 reflecting interface (TIRF). EW scattering at RI-discontinuities at or near the interface
 108 results in far-field light, too. In either case, the total intensity depends on how deep the EW
 109 reaches into the rare medium (n_1).

110 Another way to 'see' EWs is by their coupling to collective electron oscillations
 111 ('plasmons') in a thin metal film (n_2 , d_2) deposited on the glass. Momentum matching is
 112 only possible due to the foreshortening of the EW wave vector (wave-front squeezing) and it
 113 leads to a sharp decrease in the reflected intensity at the surface plasmon resonance (SPR)
 114 angle, $\theta_{\text{SPR}} > \theta_c$. Changes in the refractive index above the metal layer, n_3 , e.g., by the
 115 binding of molecules to the surface, will shift θ_{SPR} . This angle shift is the basis for SPR-
 116 based sensing, spectroscopy, and SPR microscopy of cell/substrate contact sites [9; 10; 11].

117

118 (**Figure 1** *Light confinement by an evanescent wave* close to here)

119

120 **The theoretical framework**

121 For a beam impinging in the incidence plane from the left, the EW propagates horizontally
 122 to the right. The Pointing vector \mathbf{S} of the EW is hence oriented in the same direction as the
 123 propagation direction of the refracted light at θ_c , **Fig.1A**. Its intensity decays in axial (z -)
 124 direction, with – in theory – an exponential dependence, $I(z; \theta) = I_0(\theta) \exp[-z/\delta(\theta)]$. The
 125 distance over which the intensity drops to $1/e$ (37%) of its value at the interface ($z = 0$) is
 126 called the 'penetration depth',

127

$$128 \quad \delta(\theta) = \frac{\lambda}{4\pi \sqrt{n_3^2 \sin^2(\theta) - n_1^2}} = \frac{\lambda}{4\pi n_3 \sqrt{\sin^2(\theta) - \sin^2(\theta_c)}} \quad (\text{Eq. 1})$$

129

130 It depends on the excitation wavelength λ , the polar beam angle θ and the RIs, **Fig. 1A**. We
 131 here omitted possible intermediate layers (n_j, d_j) [12; 13]. The penetration depth, δ , does
 132 neither depend on the polarization nor on the azimuthal angle ϕ of the incoming beam. On a
 133 plot of $\delta(\theta)$ vs θ (**Fig. 1B**) we recognize an asymptotic behavior, both for $\theta \rightarrow \theta_c$, for
 134 which δ diverges and becomes infinite (which is intuitive, because of the emergence of the
 135 transmitted refracted beam for $\theta < \theta_c$), as well as at very large $\theta \rightarrow \pi/2$, for which $\delta(\theta)$
 136 approaches $\lambda / (4\pi n_3 \sqrt{1 - \sin^2(\theta_c)})$, **Fig. 1C**. Thus, for a given λ , higher-index substrates
 137 (n_3) or more grazing angles θ result in a better optical sectioning.

138

139 **Many unknowns affect the true penetration depth**

140 As there is a smooth intensity roll-off, the actual penetration depth is strictly defined only in
 141 relation to a certain signal-to-background or signal-to-noise level. For a mono-exponential,
 142 $I(z)$ decays to <5% of $I(0)$ over an axial distance of two penetration depths, $z = 2\delta$. With
 143 some arbitrariness, one could thus take 2δ as the effective probe depth, because 5% is of the
 144 order of typical noise levels. Unfortunately, the question of how far excitation light reaches
 145 into the cell is more complicated, and it has been a matter of passionate debate. In practice,
 146 δ is only known within certain bounds, and often even the assumption of a single-
 147 exponential intensity decay does not hold. The reasons are the following:

148 (i), whereas λ and n_3 are identified, neither n_1 nor θ are known with much precision.
 149 For a biological cell, n_1 varies on a microscopic length-scale [14] modifying both θ_c and δ ,
 150 **Fig. 1C**. But also θ is only known with a certain accuracy (adjustment accuracy) and
 151 precision (beam divergence). These unknowns translate into an uncertainty and range of
 152 penetration depths $\delta(\theta)$, respectively.

153 (ii), even with all parameters known, the calculated penetration depth is just that:
 154 theoretical, because microscope- and sample-generated non-evanescent light modifies the
 155 intensity decay that will no longer be a simple exponential. For prismless TIRF [15] several
 156 reports have shown that excitation light distribution is best described by the superposition of
 157 a rapidly decaying and a long-range component that is fairly independent of θ [16; 17; 18].
 158 The effect of this long-range component is paradoxical: while most of the excited
 159 fluorescence is due to EW-excitation for θ just above θ_c , for very high incidence angles
 160 (that normally should produce better optical sectioning) non-evanescent light dominates.

161 (iii), background comes from stray excitation from inside the microscope objective
162 and different optical surfaces [17]. In the critical illumination scheme used in multi-angle
163 TIRF, any scatter on the scanning mirrors (or in any conjugate sample plane) is imaged into
164 the sample plane, **Figs. 2A** and **2B**, (a). Although this stray excitation can be quantitated [16;
165 17] such measurements are not routine;

166 (iv), protein-rich adhesion sites or near-membrane organelles like lysosomes or
167 dense-core granules have a higher RI than the surrounding cytoplasm. The spatial
168 inhomogeneity in $n_1(x, y, z)$ does not only affect δ directly (eq.1), but it also produces non-
169 evanescent light, **Fig. 2B**, panel (b). Scattering occurs predominantly in forward direction,
170 into angles close to the original propagation direction of the EW, parallel to the surface [17;
171 19; 20]. For even higher local $RI \geq n_1$, light can be refracted, generating intense beams in
172 forward direction, as observed in chromaffin cells packed with secretory granules [19], **Fig.**
173 **2B**, (c).

174 In view of these difficulties, authors and microscope manufacturers have resorted to
175 simply reporting a calculated $\alpha(\theta)$ using eq.(1) – see
176 <https://imagej.nih.gov/ij/plugins/tirf/index.html> for a popular ImageJ plug-in, leading to
177 overly optimistic if not unrealistic statements of light confinement at the reflecting interface.
178 The interpretation of TIRF intensities in terms of fluorophore concentration changes,
179 fluorophore axial distances or single-particle trajectories is thus often flawed by large error
180 bars or, worse, simply wrong.

181

182 **Azimuthal beam spinning does not improve axial confinement**

183 At least for illumination non-homogeneities there is remedy. A straightforward solution is 2-
184 photon EW excitation [20; 21; 22] (which is surprisingly little used). Another is azimuthal
185 beam scanning [23; 24; 25]: as scattering is directional, varying the EW propagation
186 direction scrambles both propagation and scattering directions and reduces the flare in any
187 given direction, **Fig. 2C**. ‘Spinning’ TIRF (spTIRF) or *incoherent* ring illumination [23-27]
188 reduces interference fringes and illumination non-uniformities but it does not change the
189 problem of the presence of non-evanescent excitation light [17] as it only *redistributes* and
190 equalizes intensities, **Fig. 2C**. spTIRF is increasingly being used [17; 26; 27; 28; 29; 30; 31;
191 32; 33; 34] and commercial systems are available (e.g., Leica [35], Gataca iLas [33]),
192 however, the bulk of published TIRF images has been acquired with unidirectional
193 illumination.

194 A similar effect in reducing interference fringes and obtaining a uniform illumination
 195 can be obtained with LED-powered, waveguide-based TIRF [36].

196
 197 (**figure 2** *illumination imperfections and azimuthal beam-scanning TIRF* close to here)

198 199 **Why calibrating penetration depths at all?**

200 There are a number of biophysical techniques that do *not* just aim at background rejection
 201 but **make quantitative use of TIRF intensities**, either for *localizing fluorophores* or for
 202 *measuring axial concentration profiles*:

- 203 • *Measuring axial z-distance changes of moving organelles*. One major application of
 204 TIRF has been the measurement of the topography basal plasma membrane from the
 205 reflecting interface [12, 26, 39, 48, 49] or the approach of secretory vesicles to their
 206 site of exocytosis [19, 20, 42, 45].
- 207 • *VA-TIRF*. Variable-angle TIRF (VA-TIRF) is a technique that uses systematic
 208 variations of θ for reconstructing axial fluorophore distributions, **Fig. 3A**. For a
 209 given axial fluorophore profile $C(z)$ the angle-dependent fluorescence $F(\theta)$ displays
 210 a characteristic shape. With θ known and $F(\theta)$ measured, $C(z)$ can be obtained, pixel
 211 by pixel by an inversion procedure [19; 36; 37; 38; 39; 40; 41; 42]. VA-TIRF relies
 212 on the precise knowledge of the decay constant *and* the shape of the axial intensity
 213 decay. Many studies assumed a mono-exponential intensity decay [43; 44; 45; 46;
 214 47], which perhaps holds for prism-type TIRF but seems overly optimistic for
 215 objective-type TIRF [16; 17; 32].
- 216 • *TIRF-colocalization*. Knowledge of the axial intensity decay is mandatory for multi-
 217 color excitation, **Fig. 3B**. As the penetration depth scales linearly with λ (eq.1), one
 218 can compensate the λ -dependence of δ by adjusting θ to maintain a constant probe
 219 volume in different color channels [33; 34; 48; 49]. The same applies to controls in
 220 TIR-FRET [30] with direct and FRET excitation of donor fluorescence.
- 221 • *TIRF-FRAP* [50], **Fig. 3C**, uses the combination of localized EW-photobleaching
 222 and TIRF imaging of the fluorescence recovery after photobleaching to study near-
 223 interface fluorophore mobility. The interpretation of FRAP data relies on the known
 224 probe volume. $\delta(\theta)$ calibration is even more stringent for bleaching- or photo-
 225 switching-based axial super-resolution measurements based upon consecutive VA-
 226 TIRF imaging of deeper and fluorescence deletion in more proximal layers [51].

227 Analogous arguments hold for TIRF photoactivation and photoswitching experi-
 228 ments, which include the growing group of PALM/STORM super-resolution studies,
 229 as well as optogenetic activation using EW-illumination [52].

- 230 • *TIR-FCS*. TIRF correlation spectroscopy [53; 54; 55] follows the same principles as
 231 confocal-spot FCS but gains sensitivity and surface selectivity from the additional
 232 excitation confinement, particularly when combined with confocal-spot TIRF
 233 excitation [56; 57].
- 234 • Even for less specialized TIRF applications a known penetration depth is a pre-
 235 requisite for reproducibility and for comparing data among experiments, laboratories
 236 and publications.

237

238

239 (**figure 3** *Quantitative uses of TIRF* close to here)

240

241 In the sequel, we review techniques for measuring θ and then calibrating $\delta(\theta)$ and we
 242 discuss their respective benefits and problems. We also comment on supercritical angle
 243 fluorescence (SAF) microscopy [58; 59], or “evanescence in emission” [4; 60; 61] and how
 244 it can be used either as an axial nanoscale ruler [62; 63] or be combined with TIRF to
 245 achieve a better near-membrane confinement than that obtained with TIRF alone [32].
 246 Finally, we describe a simple yet effective way for calibrating the true optical sectioning of
 247 the microscope, based on the acquisition of combined TIRF and EPI z -image stacks of a thin
 248 3-D sample of sub-diffraction beads embedded in a low-melting point agarose gel the RI of
 249 which mimics that of the cytoplasm [64].

250

251 **Measuring the polar beam angle**

252 Often, the excitation angles cover a finite range and the theoretical average of the resulting
 253 decay curves (eq.1) gives a near-exponential decay curve corresponding to the average
 254 excitation angle. Thus, it seems important to measure the polar beam angle and its spread,
 255 $\theta \pm \Delta\theta$. Most techniques for calibrating δ rely on determining θ and measuring the intensity
 256 resulting from a known axial fluorophore distribution $F(z)$. A minimal θ calibration involves
 257 measuring the positions of the setscrew or stepper motor at three characteristic points: (i),
 258 normal incidence, $\theta = 0$, epifluorescence (EPI), (ii), the critical angle θ_c and, (iii), the
 259 limiting angle θ_{NA} of the objective. Identified either from sample-plane [20] or back-focal

260 plane (BFP) images [17; 25; 46; 65], Abbe's sine condition, $r = f_{obj} n_1 \sin\theta$, is used to
 261 extrapolate to intermediate values. r is the radius in the exit pupil plane (objective BFP), and
 262 f_{obj} is the focal length of the objective, $f_{obj} = f_{TL}/M$, with f_{TL} being the focal length of the
 263 manufacturer tube lens, and M is the objective transversal magnification, **Fig. 4A**. For a VA-
 264 or spTIRF setup, one can substitute r using the focal length of the focusing lens, f_{FL} , and the
 265 substrate refractive index n_1 to obtain

$$266 \quad \theta = \sin^{-1} \left[\frac{f_{TL}}{f_{obj}} \cdot \frac{\sin(k \cdot U_\theta)}{n_1} \right], \quad (\text{Eq.2})$$

267 and k is a constant ($^\circ/V$) characteristic for the scanner used, and U_θ is the voltage applied to
 268 the polar axis of the scanner.

269 Alternatively, one can measure θ in the sample plane, with the advantage that
 270 coverslip tilt and beam misalignment are accounted for. In the 'lateral-displacement'
 271 technique, the laser is set to an oblique angle and the coverslip is topped with a drop of dye
 272 solution or a thin fluorophore film. Defocus produces a lateral movement of the fluorescent
 273 spot that can be traced from fitting a 2-Gaussian with the elliptical cross-section of the
 274 beam, **Fig. 4B**. Fitting a straight line with its center position yields $\arcsin(\theta)$ [18; 66].
 275 Alternatively, for the beam not to suffer TIR, the glass/air surface can be made transmissive
 276 by an oil-coupled external prism [25; 34] or a solid-immersion lens (SIL) [17], **Fig. 4C**, and
 277 the now refracted beam is projected to the ceiling or wall. In a variant of this triangulation
 278 technique, the back-reflected beam is picked up from the objective rear pupil and projected
 279 onto a quadrant photodiode to determine θ from the center-of-mass of a Gaussian fitted with
 280 the beam profile [67]. Similarly, taking out the emission filter permits to see the back-
 281 reflected light on a BFP image, and calculating $\theta = \arcsin[r/(n_1 f_{obj})]$ [65], **Fig. 4D**.

282 (**figure 4 Polar beam-angle calibration** close to here)

283 284 **Intensity-based measurements of evanescent-wave penetration depths**

285 Once a look-up table for θ has been generated, we can adjust θ and estimate $\delta(\theta)$. This
 286 involves localizing the reflecting interface and measuring the fluorescence (or some other
 287 variable) for different fluorophore heights. This procedure is repeated for several incidence
 288 angles θ and the obtained curve compared with the calculated one (fig. 1B).

289 a) *Calibration samples with a known axial fluorophore profile.* **Fig. 5** shows examples of
 290 such samples, including the “raisin cake”, a random, sparse 3-D distribution of sub-
 291 diffraction fluorescent microspheres in an index-matched gel, **Fig. 5A**. The acquisition of a
 292 z -stack of images can localize these beads with respect to the reflecting interface [64].
 293 Alternatively, point emitters can be fixed to the surface of an oblique microscope slide, **Fig.**
 294 **5B**, [33], a large convex [68] or concave lens [65]. Equivalently, one can use the contour of
 295 an index-matched dye-coated large-diameter fluorescent bead, or an unlabeled bead
 296 embedded in dye-containing medium [16], **Fig. 5C**. An elegant *in cellulo* variant is the use
 297 of a tilted fluorescently labeled microtubule [69].

298 All the above test samples have in common the requirement for an evenly lit field of
 299 view (see [17] for the limits of this approximation) and they all need z -scans to locate the
 300 fluorophores with respect to the reflecting interface. This adds a complication for objective-
 301 type TIRF as the reflected beam displaces laterally upon focusing (see above), and the z -
 302 dependence of the PSF (detection volume). Additionally, z -dependent spherical aberrations
 303 require a correction, see [69] and our calibration protocol, below.

304 b) *Semi-infinite dye layers.* Simpler is the use of VA-TIRF and a thick, $d \gg \delta(\theta)$,
 305 homogenous fluorescent sample [20; 38] to estimate the effective penetration depth from the
 306 variation of the *cumulative* fluorescence, **Fig. 5D**. Assuming a two-component axial decay,
 307 i.e., the sum of mono-exponentially decaying EW with a decay length $\delta(\theta)$ and a long-range
 308 component with $D \gg \delta$ [16; 18] we express the measured fluorescence as

$$309 \quad F(z) = b + A \cdot e^{-z/\delta(\theta)} + B \cdot e^{-z/D} \quad (\text{Eq.3})$$

310 Here, we assumed that D to be only slowly varying with θ and the offset b to be negligible.
 311 After integration over z in the bounds $[0, \infty]$, eq.3 yields a linear dependence of the
 312 measured fluorescence on $\delta(\theta)$,

$$313 \quad F_{\text{tot}}(\theta) = A \cdot \delta(\theta) \cdot \left(1 - e^{-\frac{z}{\delta(\theta)}}\right) + B \cdot D \cdot \left(1 - e^{-\frac{z}{D}}\right) \approx A \cdot \delta(\theta) + B, \quad (\text{Eq.4})$$

314 because the second term is an angle-independent offset. If eq.4 is normalized for the θ -
 315 dependence of the incident intensity at the interface, $I_0(z=0)$, e.g., by recording $F_0(\theta)$ of a
 316 thin fluorophore film at the interface [20; 40; 70], then the implicit θ -dependence of A and B
 317 is cancelled out and $\delta(\theta)$ can be obtained from the slope on a plot of the measured $F_{\text{tot}}(\theta)$

318 against $F_0(\theta)$. Of note, for the integral to solve as indicated we had to assume that the θ -
319 dependent term of the intensity decay follows a mono-exponential.

320 *c) Fluorescence correlation spectroscopy (FCS).* Another way to determine the axial
321 confinement is TIR-FCS [53; 54; 55]. The intensity fluctuations resulting from single
322 molecules moving in and out of the excitation volume are being used to estimate its size,
323 **Fig. 5E**. Again assuming a mono-exponential intensity decay, one can explicitly solve the
324 autocorrelation function and back-calculate the dye concentration, the bulk diffusion
325 coefficient and the excitation volume [53]. Complications can arise from dye adsorption at
326 and hindered mobility near the interface, from the distance-dependent fluorescence
327 collection efficiency [38] as a consequence of the change in the fluorophore radiation
328 pattern for interface-proximal dipoles (see below) and, of course, from a non-exponential
329 axial intensity decay.

330
331 *d) Single-spot measurements.* Other approaches measure $\alpha(\theta)$ only in one point, either by
332 sampling the *local* EW intensity with a thinned optical fiber tip connected to a photodetector
333 [71], by measuring the fluorescence generated by a sub-resolution fluorescent bead fixed at
334 the tip of an AFM cantilever [72; 73; 74] or the tip of a micropipette [43]. Brutzer *et al.* used
335 a four-arm DNA junction as a nanomechanical translation stage to propel a single
336 fluorescent quantum dot through EW field [75], **Fig. 5F**. A similar strategy uses a
337 combination of magnetic tweezers and a supercoiling DNA together with a fluorescent
338 nanodiamond-labeled magnetic bead and surface-immobilized fluorescent nanodiamonds as
339 a reference for $z = 0$ [76]. Alternatively, fluorophores at different distances z could be
340 obtained with static 3-D scaffolds, e.g., tetrahedral DNA-origami fluorescent rulers of
341 different dimensions [77; 78; 79]. These approaches have the advantage that they move the
342 fluorophore through the field so that the focus can remain at a single, near-interface layer,
343 but, by definition, they are limited to single-point measurements not representative of what
344 happens across the field of view.

345 (**figure 5** close to here)

346
347 *e) Index-matched polymer steps.* Many of the above approaches either perturb the EW by
348 the presence of RI boundaries (i.e., the edge of a glass slide, a lens touching the interface, or
349 a fiber tip), they require tedious sample preparation, or they are not applicable in the

350 aqueous environment of biological TIRF. Taniguchi's group introduced a calibration slide
351 featuring steps of different nanometric heights fabricated from a non-fluorescent polymer,
352 the RI of which matched that of water. This spacer staircase was topped with sub-diffraction
353 fluorescent microspheres generating fluorescent steps at different distances from the
354 reflecting interface [80; 81], **Fig. 6A**, *left*. The Schwille lab recently proposed a variant that
355 used - instead of combining several nanofabrication techniques as in the Unno papers - a
356 simpler dip-coating method in which steps are produced by successively dipping a glass
357 slide into different depth of a polymer solution. They simply topped their staircase polymer
358 with AlexaFLuor488 solution. They then measured the EW-excited fluorescence as a
359 function of step height [18], reminiscent of the "infinite dye layer" technique, **Fig. 6A**, *right*.
360 Corroborating earlier work, both studies confirmed that the axial intensity profile contained
361 both evanescent and non-evanescent contribution and that the effective penetration depth
362 generally exceeded the calculated one.

363 A yet different approach uses thin films of optically transparent index-matched
364 polymers on glass substrates wrapping a nanometric dye layer. **Fig. 6B** illustrates such a
365 'sandwich' consisting of a non-fluorescent spacer layer that onto which J-aggregates were
366 electrostatically adsorbed, resulting in a ~2-nm thin, homogenous emitter layer, **Fig. 6B**,
367 *inset*. This layer was finally spin cast with another, 5- μm thick, polymer layer to produce a
368 uniform dielectric environment [82]. The advantage of this multi-layer test sample is that it
369 produces a thin, controlled and homogenous dye distribution at a precisely controlled
370 distance from the reflecting interface. With several of such test samples, plotting the
371 collected fluorescence as a function of the fluorophore distance Δ allows the measurement
372 of the axial effective intensity decay, **Fig. 6C**.

373
374 ([figure 6](#) close to here)

375

376

377 **Other descriptors than intensity**

378

379 Until now, we have focused on techniques for calibrating the EW that relied on fluorescence
380 intensity measurements from test samples having a known axial fluorophore profile.
381 However, other parameters of fluorescence can be used, too.

382

383 *Fluorophore radiation pattern.* Fluorophores change their radiation pattern when they
384 approach a RI boundary closer than $\sim\lambda$ because the evanescent component of their radiation
385 can couple to the interface, become propagative [83; 84; 85] and detectable in the far field at
386 angles $>\theta_c$ ‘forbidden’ by Snell’s law. The selective detection of this ‘super-
387 critical angle fluorescence’ (SAF) [60; 86; 87] features a similar surface selectivity and
388 background suppression of TIRF whilst not requiring EW excitation. As SAF probes the
389 same sub- λ length-scale, it can be used in conjunction with EW-excitation for achieving a
390 better surface selectivity than TIRF alone [32]. Its improved optical sectioning results from
391 the filtering out of signal originating from deeper sample layers that are incapable of
392 *emitting* light into “forbidden angles”, and a secondary effect is that the *detected*
393 fluorescence also decays steeper than the excitation decay because for fluorophores nearer
394 to the surface, increasing amounts of SAF are captured. For a given objective-NA and
395 sample refractive-index, this translates into the distance-dependent collection efficiency
396 [40]. (SAF requires high-NA collection from the glass side and hence does not work with
397 upright detection from the far side).

398 Fourier-plane imaging conveniently measures the radiation pattern [88]. The BFP
399 image has other benefits for calibrated TIRF microscopy: it allows determining the cell’s
400 near-membrane RI, n_1 , from the radius at which the transition between SAF and
401 undercritical angle fluorescence (UAF) occurs [14; 61; 89]. The SAF/UAF intensity ratio is
402 proportional to fluorophore height [4; 61; 62; 89] a feature, which can be used for combined
403 axial fluorophore localization and penetration-depth calibration by plotting the total
404 fluorescence SAF+UAF vs. (SAF/UAF) [82] (**Fig. 6C**).

405
406 *Fluorescence lifetime*, τ is a measure of how long the molecule stays in the excited state
407 after absorption of a photon. τ is one over the sum of the radiative and non-radiative decay
408 rates. Fluorescence lifetime oscillations and shortening are observed in the presence of a
409 metal coating [90; 91], a near-field probe tip [92] or a metal nanoparticle [93]. For the sub- λ
410 distances relevant here, non-radiative energy transfer from the excited molecule to the metal
411 offers an alternative decay path for excited-state relaxation. Distance-dependent surface
412 quenching by a thin gold layer was used to measure fluorophore heights of dye-labeled
413 microtubules [94] on the basis of the model by Chance, Prock, and Silbey (CPS model) that
414 relates τ to the fluorophore height z [90].

415 While the distance-dependent lifetime quenching is strong for metal, a much smaller
416 effect is observed on bare glass [84]. Also, other factors than surface distance interfere with
417 molecular lifetimes, including the orientation [95], local RI [96], solvent polarity, viscosity
418 as well as complex formation and collisions in the presence of nearby quenchers.

419
420 *Other*, more exotic ways for axially localizing particles and calibrating $\delta(\theta)$ include inter-
421 ferometric, PSF-engineering-based, axially-structured illumination-based or multi-plane
422 based axial super-resolution techniques (reviewed in [97]), but these generally require
423 considerably more complex instrumentation. Yet other approaches use not light but other
424 physical parameters that are modified by the presence of an interface, e.g., the anisotropic
425 diffusion near an interface [98] or the flow velocity gradient of particles moving under the
426 influence of a rotating disk [99] to calibrate the EW decay.

427
428 In summary, experimenters dispose of a host of observables for estimating the axial
429 intensity decay at or near a dielectric interface. In spite of a common quest for quantitative
430 TIRF imaging, neither a consensual test sample, nor common metrics, nor an industry
431 standard has emerged for calibrating evanescent-wave excited fluorescence intensities.
432 Instead, different labs have come up with their own customized solutions, making the
433 comparison among studies difficult, if not impossible. In the now dominantly used prismless
434 objective-type TIRF, the quantitative interpretation of TIRF intensities is problematic due to
435 the co-existence of a localized, evanescent and a non-evanescent, long-range excitation
436 component that has consistently been observed using different protocols [16; 17; 18] and
437 that would call for a systematic approach to better compare among experiments, data sets
438 and setups. Furthermore, at the same beam angle and beam diameter, the same TIRF
439 objective, used in conjunction with different illumination optics, will perform differently.
440 Conversely, in our lab, we observed that nominally identical objectives performed
441 differently on the same microscope, pointing to important fabrication tolerances for these
442 high-NA objectives when used at the very limits of their NA. It would thus seem important
443 that the field agrees on a protocol for testing, evaluating and quantifying axial confinement
444 in EW techniques.

445 446 **A simple protocol for interpreting, comparing, and sharing TIRF data**

447 A reference standard for calibrating the effective probe depth in TIRF and SAF micro-
448 scopies should meet the following requirements, it

- 449 (i) should work in an aqueous environment mimicking the cytoplasm; specifically,
450 it should not perturb the EW by introducing objects having a different refractive
451 index,
- 452 (ii) should not require modifications to existing TIRF and SAF setups,
- 453 (iii) should be easily transposable from one lab to the other, be stable or, alternative-
454 ly, easily and reproducibly to fabricate,
- 455 (iv) should mimic the conditions at the dielectric interface typically encountered in
456 biological TIRF microscopy, i.e., it should work close to the refractive index of
457 the intracellular environment, and,
- 458 (v) should be compatible with different TIRF geometries to permit comparisons
459 among setups and experiments.

460

461 We advocate the ‘raisin cake’ method for measuring the effective penetration depth in a
462 sparse 3-D fluorophore distribution. Our method [100] is based on commercially available
463 fluorescent microspheres located on the glass/water interface and at different axial positions
464 in a transparent, index-matched agarose gel. It does not require any specific equipment other
465 than that available in any wet lab. In as much as the calibration procedure involves the
466 acquisition of z -stacks of images, a precise focus drive is needed for accurate focusing and
467 for quantifying the effect of defocus.

468 The principle of the measurement is to first localize beads axially. For each bead, its
469 location is obtained by fitting a Gaussian distribution with the measured axial fluorophore
470 profile, $F(z)$. This fit yields both the peak fluorescence and the corresponding axial bead
471 position. Next, to account for variability in intrinsic bead fluorescence (or several beads
472 sticking together), the same z -stack of images is acquired upon EPI excitation, assuming a
473 negligible z -modulation of the excitation intensity. The in-focus EPI intensity is used for
474 normalization at z_0 , the plane at which near-surface beads lit by the EW appear in focus.
475 This normalization is applied both to the measured bead intensities of the EPI and the TIRF
476 image at z_0 , so that the intensity modulation resulting from the modulation of the collection
477 of the emitted fluorescence due to the limited depth-of-field of the objective and that
478 resulting from the excitation confinement upon TIRF can be separated.

479 100-nm TetraSpecksTM beads were deposited on a coverslip and at different axial
480 distances from the coverslip by embedding them in a low-density agarose solution
481 containing sucrose to increase the refractive index to 1.374 (see Appendix for details). We
482 first acquired an epifluorescence (EPI) image stack across the sample. The individual image

483 planes were spaced by $\Delta z = 10$ nm, covering a range from -1.0 μm below to $+2$ μm above
 484 the reflecting interface. For TIRF, we used azimuthal beam spinning to even out
 485 illumination heterogeneities, **Fig. 7A**. Acquisitions were realized with autofocus feedback to
 486 exclude focal drift during recordings. We determined the axial location of each bead by
 487 measuring the background-subtracted intensity in a 3×3 pixel region of interest (ROI)
 488 centered on each bead, see **Fig. 7B, inset**, and plotted it against z . **Fig. 7B** shows examples
 489 of axial fluorescence profiles $F(z)$ of two beads located at different z -positions. We fitted
 490 Gaussian distributions with $F(z)$ and chose the peak location of the Gaussian as the bead
 491 position. Similar to the sub-diffraction xy -localization precision in PALM or dSTORM, this
 492 method allowed us to determine bead positions with 15-nm precision, well below the axial
 493 resolution of the objective. 100-nm diameter beads gave more consistent results than 200-
 494 nm beads (not shown), but even for the smaller beads the axial intensity profile did not
 495 always follow a Gaussian distribution. In some cases impurities or microbubbles in the
 496 agarose, low signal-to-noise or the superimposition of the images of beads close by distorted
 497 the profile, **Fig. 7B, right**. Also, scattering from neighboring polystyrene beads may
 498 contribute. Asymmetry also arises for beads that are directly on the coverslip because the
 499 vicinity to the glass/medium interface distorts the axial intensity profile due to spherical
 500 aberration at the RI-boundary. We therefore eliminated all beads that displayed a non-
 501 Gaussian profile from analysis.

502

503 ([figure 7 Raisin-cake calibration](#) close to here)

504

505 Focusing at the bead layer that appeared in-focus upon TIRF-illumination but then
 506 switching to EPI, we acquired for each bead $F_{\text{EPI}}(z_0)$. EPI illumination was rapidly
 507 interleaved with VA-TIRF by steering the galvos to $(0, 0)$, so that the incident beam
 508 propagated on the optical axis. This EPI image acquired at z_0 divided by the bead's in-focus
 509 intensity $F_{\text{EPI}}(z_0)/F_{\text{EPI}}(z)$ allowed us to assess the influence of the intrinsic fluorescence of
 510 the bead, and of the depth-of-focus of the objective on the fluorescence intensity of beads
 511 remote from the interface, **Fig. 7C**. Plotting the thus normalized fluorescence intensity
 512 against the previously determined axial bead position z , we found that $F_{\text{EPI}}(z_0)/F_{\text{EPI}}(z)$
 513 decayed with a length constant of 481 ± 166 nm from the z_0 , **Fig. 7D**. The same intensity
 514 envelope will also modulate the bead intensities in TIRF and be convoluted with additional
 515 fluorescence decay resulting from the intensity decay of the EW. On **Fig. 7E** this effect is
 516 seen, when plotting the axial intensity decay of the beads upon EW excitation at different

517 polar beam angles θ , after normalization. Indeed, $F_{\text{TIRF}}(z_0)/F_{\text{EPI}}(z)$ was best described by the
 518 superposition of a long-range and short-range exponential decay (Eq.3). As expected, we
 519 found that the remaining short-range component was modulated by changing θ when setting
 520 the long-range component to the earlier measured EPI decay ($D = 481 \pm 166$ nm). Focusing
 521 at the bead layer that appeared in-focus upon TIRF-illumination (z_0) we extracted the
 522 fluorescence intensity of all beads in the field of view at different polar angles between
 523 67.5° and 72.5° . For each bead, $F_{\text{TIRF}}(z_0)/F_{\text{EPI}}(z)$ was plotted against its z -position and a
 524 double exponential fitted with the ensemble of data points from all beads. We took the
 525 short-range component as the effective penetration depth $\delta(\theta)$. At $\theta = 67.5^\circ$, 70.0 and 72.5° ,
 526 the obtained $\delta(\theta)$ was (139 ± 20) nm, (109 ± 16) nm, and (91 ± 13) nm, respectively, close
 527 to the calculated penetration depths of 134, 100 and 84 nm, respectively. These values were
 528 calculated according to eq. 1, assuming n_1 and n_3 as 1.374 and 1.52, respectively, and using
 529 an interpolated position LUT generated previously during calibration of beam angle in terms
 530 of galvo positions for θ . At $\theta = 67.5^\circ$, 70.0 and 72.5° , the decay lengths measured from
 531 short-term components are, respectively 4%, 9% and 8% larger than theory.

532 At $\theta = 65^\circ$, just above $\theta_c = \text{asin}(1.374/1.52) = 64.7^\circ$, fitting a single- or double
 533 exponential with the measured fluorescence intensity decay similarly resulted in $\delta(65^\circ) =$
 534 367 ± 217 and 349 ± 118 nm, respectively, indicating that non-evanescent stray excitation
 535 impaired the penetration depth estimation, compared to a calculated $\delta_{\text{calc}}(65^\circ) = 319$ nm.

536 Consistent with earlier reports [16; 25; 32], we find that supercritical-angle
 537 illumination through the periphery of a high-NA objective produces a mix of evanescent and
 538 EPI illumination. For beam angles of 72.5° , 70.0 and 67.5° , the fractional weight $B/(B$
 539 $+A+B)$ from eq.(3) of non-evanescent excitation light amounted to 13, 15.3, and 15.5%.
 540 However, to which degree of evanescent vs. non-evanescent light a fluorophore is exposed
 541 to, will depend on its very distance from the reflecting interface and, paradoxically, the
 542 contribution of non-evanescent light much exceeds the above percentages for $z \gg 0$,
 543 because the inhomogeneous EW field decays steeply while the EPI component remains
 544 relatively more important.

545 Taken together, our technique permits a reliable measurement of the effective axial
 546 confinement at the reflecting interface and a quantification of the relative amount of
 547 evanescent and non-evanescent excitation components. Its accuracy depends on the axial-
 548 localization accuracy of the beads, which in turn is a function of amount of signal available
 549 (this is why we localize beads in EPI rather than TIRF). In our experience, the same

550 protocol is applicable to fixed cells, in which small organelles are labeled (data not shown).
551 In this case, the density of labeled organelles has to be low to avoid the superimposition of
552 fluorescent structures. Furthermore, the object size and their fluorescent intensity have to be
553 fairly homogenous to avoid artifacts. Autofluorescence, e.g., of lysosomes or lipofuscin-rich
554 granules can compromise the in-cell estimation, because these organelles often cluster
555 together.

556
557

558 **Conclusions**

559 *Origins of non-EW excitation light.* Quantitative TIRF techniques generally have to assume
560 not only a known penetration depth but also a single-exponential EW field decay. We argue
561 in this paper that this assumption is rarely met in a typical biological experiment. In
562 objective-type TIRF, excitation impurities have three major sources, (i), light scattered at
563 the edges of the high-NA objective due to its use at extreme angles close to the limiting
564 aperture [16]; (ii), a residual long-range epi-fluorescence component that emerges from deep
565 inside the microscope, typically from the beam scanners that are imaged by a relay telescope
566 into the sample plane [17; 18]; (iii), the fluence of EW is also influenced by surface
567 scattering at the interface (clumps of adhesion molecules, scratches, focal adhesion sites)
568 and the distribution of RI heterogeneity and scatterers in the medium inside the cell
569 (organelles, cytoskeleton, ...). In a recent study we found that this component (at least with
570 cultured astrocytes, fibroblasts and neurons) is not the dominant source [17] compared to
571 instrument-induced imperfections, but our older work with bovine adrenal chromaffin cells
572 [45] or PC12 cells illustrates cases where cell-induced scattering is highly relevant.

573

574 *Possible Remedies.* Prism-based TIRF (see, e.g., Ref. [47]) provides ‘cleaner’ and more
575 uniform illumination as well as a higher-accuracy angular positioning, therefore it should be
576 applied where an accurate intensity profile is of importance. Point-scanning TIRF [58]
577 (albeit slower) has slightly better lateral resolution than wide-field TIRF and can be
578 combined with SAF detection [63] to improve axial confinement. Non-linear EW excitation
579 [20; 21] effectively eliminates non-EW light by way of confining fluorescence excitation to
580 the volume where the photon density is high enough to support the non-linear absorption
581 process.

582 Independent of the very approach used, a variety of EW-calibration techniques exist, among
 583 which we advocate the simple, inexpensive and reproducible ‘raisin-cake’ technique, that
 584 involves the following steps,

- 585 • calibrate the polar beam angle θ , either by ray tracing through the illumination
 586 optical path or via triangulation after the passage of the beam through the objective;
- 587 • Use SAF refractometry with the same fluorophores you plan to use for TIRF imaging
 588 to get an idea of the average refractive index, $\langle n_1 \rangle$, surrounding the label;
- 589 • Use polar beam angles that are neither too close to the critical angle nor too close the
 590 edge of the objective NA;
- 591 • Use θ -sweeps to study how different angles affect the TIRF image;
- 592 • Use azimuthal beam spinning TIRF to homogeneously illuminate the scene;
- 593 • Calibrate the effective penetration depth using the ‘raisin cake’ technique;
- 594 • Systematically report $\theta \pm \Delta\theta$, $\langle n_1 \pm \Delta n_1 \rangle$, and the estimated $\delta \pm \Delta\delta$;
- 595 • Combine TIRF excitation and vSAF detection for better optical sectioning;
- 596 • Discuss, how the uncertainty in δ and TIRF intensities affects your conclusions.

597
 598 Of course, our method for estimating $\delta(\theta)$ uses a reduced model system that consists of
 599 uniformly sized beads in a homogeneous medium. Polystyrene and agarose have uniform,
 600 but different RIs. In reality, (i) different cell types/cell cycles/organelles/vesicles have
 601 different RI and diameters and act as a heterogeneous population of scatterers; (ii), uniform
 602 fluorescence staining of targets, and their distribution cannot be controlled, so that even for
 603 equal pixel fluorescence, the (sub-diffraction) organelle size and local dye concentration add
 604 other unknowns. In principle these unknowns can be disambiguated with VA-TIRF, but in
 605 practice this offers little help, as the data analysis implicitly assumes a single-exponential
 606 EW field decay [39-43; 48; 49].

607 We believe that despite its limitations, the method suggested here - together with a
 608 systematic framework to report the results and its uncertainties - will provide a reasonable
 609 estimate of EW penetration in the absence of further complications, and it will facilitate
 610 comparisons of results between images, experiments, and research groups.

611 **APPENDIX A: TIRF test-sample protocol**

612 *Rationale.* In the following appendix, we present a reliable straightforward, and relatively
613 simple protocol for measuring $\delta(\theta)$. Like the alternative ‘big lens’ experimental depth
614 determination (Ref. [16]), our technique has the advantage of using measurements across the
615 entire field of view. Our technique relies on a two-step calibration, (i) a precise knowledge
616 of the beam angle and its spread, $\theta \pm \Delta\theta$, and, (ii), the calibration of the local fluence of the
617 exciting wave field by a large series of point measurements at different axial distances. This
618 protocol accompanies the experimental data shown in **Fig.7**.

619 For users that will not have precise angle information the same strategy can be used
620 in a non-quantitative manner, by preparing a series of sandwich-slides, drop-cast with a
621 mono-layer of 100-nm beads, covered with a non-fluorescent spacer layer (agarose devoid
622 of beads), which is then topped with the standard bead-containing layer. This only requires a
623 piezo-focus drive, and one can then determine *up to which distance from the reflecting*
624 *interface (localized by the first monolayer of beads) fluorescence is still excited.* In this case,
625 the answer will not be a calibration curve but an ON-OFF-type information. For example, if
626 beads are seen in EPI 500 nm above the interface but they disappear upon TIRF, then the
627 EW field has decayed to undetectable levels over 500 nm.

628
629 *Sample preparation.* Small droplets of a 1:4 dilution in water of 0.1- μ m diameter
630 TetraspeckTM polystyrene beads (Invitrogen) were deposited on a glass coverslip (BK-7,
631 e.g., FisherScientific or Marienfeld) and allowed to dry, **Fig. S1A**. The bead surplus was
632 flushed with water. In parallel, a solution of 0.5% low-melting point agarose solution
633 (seaplaque GTG agarose, Lonza) containing 842 mM sucrose was prepared. Sucrose
634 increased the RI of the solution to 1.375 to mimic the refractive index of cells [101]. The
635 solution was heated to $\sim 80^\circ\text{C}$ in a water bath to melt the agarose. It was then cooled down
636 and kept at a constant $\sim 50^\circ\text{C}$. 5 μ l of sonicated beads were transferred to a pre-heated
637 Eppendorf tube to which 15 μ l agarose/sucrose solution was added and thoroughly triturated
638 with a preheated pipette. The now 20 μ l of solution was pipetted onto the coverslip at the
639 same location, topping the beads that were previously attached to the glass (**Fig. S1B**). The
640 agarose was allowed to polymerize. To speed up this step the coverslip can be placed on an
641 ice-cold surface. Then, 1-2 ml agarose/sucrose solution (not containing beads) was slowly
642 deposited on top of the bead-containing drop to achieve a thick homogeneous layer of RI =
643 1.375. We chose this value based on literature values and our own SAF-based measure-

644 ments of the RI of the near-membrane space [14]. The RI of jellified agarose was
645 determined on an Abbe refractometer. Although not indexed-matched, the influence of the
646 TetraSpeck microspheres (RI ~ 1.6 , in the range of 1.59-1.64 at 488nm with important
647 variations among individual beads, [104]) in the agarose gel is relatively negligible, because
648 their density is very low (so that neighboring PSFs did not overlap) and their scattering
649 anisotropy of ~ 0.1 [105] mainly gives rise to a complete redistribution of angles-of-flight of
650 scattered photons and hence result in a faint, diffuse background rather than pronounced
651 cones of forward-scattered light.

652 To obtain reproducible results, it is important that the topping agarose solution is
653 maintained at a temperature just above the gelling point of agarose ($\sim 30^\circ\text{C}$) so that the
654 small bead-containing drop already on the coverslip does not re-melt when the large volume
655 of agarose/sucrose solution is added.

656
657 *Setup.* The penetration depth was measured on a commercial multi-angle objective-type
658 TIRF setup (Visitron Systems, Puchheim, Germany) built around an IX83 inverted
659 microscope equipped with an autofocus module, and a UAPON100XOTIRF NA-1.49
660 objective (all Olympus). Precise focusing was achieved with the motorized focus drive ($\Delta z =$
661 10 nm). For both EPI and TIRF illumination, the beam of a 488-nm 100-mW laser was
662 directed into the iLAS² beam scanning system (Gataca Systems, France) allowing (θ, ϕ)
663 scans. Fluorescence was collected through the same objective, extracted with a
664 ZT405/488/561/640rpc multi-band dichroic, a ZET405/488/561/640rpc multi-band emission
665 filter (both from Semrock) and detected on an Evolve-EM515 electron-multiplying charge-
666 coupled device (EMCCD) camera (Photometrics). All setup components were controlled by
667 VisiView software (Visitron Systems GmbH). The resulting pixel size in the sample plane
668 was 160 nm. Typical integration times were 100 ms at a hardware gain 1 and an EM gain of
669 200 arbitrary units.

670
671 *Data analysis.* Data points (bead fluorescence vs axial position) were determined from two
672 series of axial (z -) stacks. Images were analyzed with imageJ (Rasband, W.S., ImageJ, U. S.
673 National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-
674 2014). Fitting and further analysis of the data was performed with IGOR PRO software
675 (Wavemetrics, Lake Oswego, OR, USA).

676

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688

689 Author contributions

690

691 MO, UB, MB, AS, and AW performed experiments, MB, UB and AW prepared samples,
692 MO, UB and AS analyzed the data, MO wrote the manuscript with contributions from all
693 authors. All authors have given their approval to the final version of the manuscript.

694

695 Conflicting interest statement

696

697 The authors declare no conflict of interest.

698

699

700 **List of abbreviations**

| | | | |
|-----|-----------|---|---|
| 701 | | | |
| 702 | DNA | - | desoxy-ribonucleic acid |
| 703 | EPI | - | epifluorescence |
| 704 | EGFP | - | enhanced green fluorescent protein |
| 705 | EW | - | evanescent wave |
| 706 | FCS | - | fluorescence correlation spectroscopy |
| 707 | FRAP | - | fluorescence recovery after photobleaching |
| 708 | NA | - | numerical aperture |
| 709 | PALM | - | photoactivation localization microscopy |
| 710 | PSF | - | point-spread function |
| 711 | ROI | - | region of interest |
| 712 | SAF | - | supercritical angle fluorescence |
| 713 | SIL | - | solid immersion lens |
| 714 | SIM | - | structured illumination microscopy |
| 715 | SMD | - | single molecule detection |
| 716 | SOFI | - | superresolution optical fluctuation imaging |
| 717 | SPR | - | surface plasmon resonance |
| 718 | spTIRF | - | spinning TIRF |
| 719 | STED | - | stimulated emission depletion |
| 720 | STORM | - | stochastic optical reconstruction microscopy |
| 721 | TIR(F(M)) | - | total internal reflection (fluorescence (microscopy)) |
| 722 | UAF | - | undercritical angle fluorescence |
| 723 | VA-TIRF | - | variable-angle TIRF |

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987 FIGURE LEGENDS

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990 FIGURE 1. *Fundamentals of TIRF excitation.* (A), *left*, prismless (objective-type) TIRF. A laser
991 beam is focused to an eccentric position in the back-focal plane (BFP, *dashed* line) of a high-
992 numerical aperture (NA) objective, generating a collimated beam impinging at an oblique angle θ at
993 the dielectric interface (*solid grey*, $n_3 > n_1$). *Middle*, for θ exceeding the critical angle, $\theta_c = \text{asin}(n_1/n_3)$,
994 the beam is totally reflected at the interface and an inhomogeneous surface wave is generated in the
995 rare medium (n_1). This ‘evanescent’ wave (EW) propagates along the surface (the Pointing vector,
996 \mathbf{S} , is oriented in $+x$ direction for a beam impinging from the left, *red arrow*), and its intensity decays
997 exponentially with axial ($+z$) distance from the reflecting interface, *right*, with a length constant
998 (‘penetration depth’) δ of the order of 100 nm. (B), dependence of δ on θ , for $\lambda = 488$ nm, $n_1 = 1.35$,
999 and for different substrate indices n_3 . The higher n_3 the smaller the critical angle θ_c and the better the
1000 optical sectioning. For a typical borosilicate glass/cell-interface and a NA-1.45 objective, the maxi-
1001 mally attainable angle θ_{NA} limits the penetration depth to 73 nm (*red dash*). In this angle range, δ
1002 depends steeply on θ , demanding high precision and accuracy when adjusting θ . (C), dependence of
1003 θ_c and δ_∞ on substrate index, n_3 . The asymptotes of the critical angle θ_c and limiting penetration
1004 depth δ_∞ for grazing incidence ($\theta \rightarrow 90^\circ$), respectively, decrease monotonously with n_3 . *Red* line
1005 indicates $n_3 = 1.52$ (BK-7), as earlier.
1006

1007 FIGURE 2. *Azimuthal beam scanning evens out illumination imperfections.* (A), schematic layout
1008 for polar- and azimuthal beam scanning TIRF (‘spinning’ TIRF, spTIRF). A 2-axis scan (θ , ϕ) is
1009 relayed via a $4f$ beam compressor (BC) into an equivalent sample plane (ESP) and - via the
1010 telescope formed by the focusing lens (FL) and objective (obj) – imaged into the sample plane (SP).
1011 *Inset* shows light distribution in the back-focal plane (BFP). Abbreviations: dic – dichroic mirror,
1012 EMCCD – electron-multiplying charged-coupled device camera, EBFP – equivalent back-focal
1013 plane, SD – scanning device. Due to this critical illumination any illumination imperfections in
1014 ESP’ and ESP show up in SP. (B), sources of non-evanescent excitation, (a), dust or scratches on
1015 the scanning mirrors and in intermediate sample planes are directly imaged into the sample plane,
1016 resulting in illumination impurities and glare; (b) EW scattering at refractive-index (RI) boundaries
1017 produces light propagating in forward direction, modifying the effective $\delta(\theta)$ across the field-of-
1018 view; (c), for shallow angles θ just above θ_c , protein-rich cell adhesion sites can have a RI high
1019 enough to disrupt total reflection and generate intense beams of refracted light. (C), negative-
1020 staining experiment, in which a non-labeled BON cell was embedded in fluorescein-dextran
1021 containing extracellular saline and imaged in unidirectional TIRF (*top*) and spTIRF (*bottom*),
1022 respectively (from [32]). The bottom of the cell adhering to the coverslip excludes the extracellular
1023 dye and appears as a dark ‘footprint’. Note the flare in EW propagation direction (*top*), which is
1024 abolished upon spTIRF (*bottom*).
1025

1026 FIGURE 3. *Quantitative uses of TIRF.* (A), variable-angle (VA-) TIRF. Smaller θ translate into
1027 larger illumination depths $\delta(\theta)$ (block arrow) allowing a topographic reconstruction of axial
1028 fluorophore profiles from a multi- θ stack. (B), multi- λ excitation. δ scales linearly with λ (block
1029 arrows). Toggling between different excitation wavelengths alters the excited volume (grey

1030 arrowheads), *top*. Simultaneously adjusting θ between different-color acquisitions maintains a
 1031 constant excitation volume, permitting quantitative co-localization or FRET studies at or near the
 1032 basal plasma membrane, *bottom*. (C), TIR-FRAP, a variant of fluorescence recovery after
 1033 photobleaching, sequentially images, bleaches and re-images a sample with EW excitation. An
 1034 intense pulse of evanescent light (flash) selectively bleaches the surface-proximal fluorophores.
 1035 Unbleached molecules from deeper sample regions repopulate the bleached volume. TIRF-FRAP
 1036 allows studying the average mobility and mobile fraction of near-membrane fluorescent species,
 1037 *inset*. Here, the penetration depth is constant, and the illumination intensity is modulated between
 1038 imaging and bleaching episodes.

1039
 1040 FIGURE 4. *Incidence-angle calibration*. (A), the radial position r of a focused laser spot in the BFP
 1041 unambiguously determines the beam angle θ . Three characteristic points can be easily identified:
 1042 (1), epifluorescence (EPI) at normal incidence, $\theta = 0 \Leftrightarrow r = 0$; (2) the disappearance of the refracted
 1043 beam at $r_c, \Leftrightarrow \theta_c$ (TIR) and, (3) the disappearance of the reflected beam at r_{NA} , when the incident
 1044 beam is beyond the limiting numerical aperture of the objective $\theta_{NA} = \arcsin(NA/n_1)$. Intermediate
 1045 values of $\theta(r)$ are interpolated from Abbe's sine condition, $r = f_{obj} n_1 \sin(\theta)$. (B), lateral-
 1046 displacement assay. The beam is directed at an oblique angle against an oil-coupled coverslip,
 1047 coated with a thin fluorophore layer, *green*. Depending on the amount of defocus, the beam
 1048 intersects the coverslip at different positions, and the lateral offset of the fluorescent spot on the
 1049 camera image, together with the knowledge of the piezo-controlled defocus permits to triangulate θ .
 1050 A similar strategy is used in (C) for $\theta > \theta_c$. Here, an oil-coupled, index-matched solid immersion
 1051 lens (SIL) prohibits TIR and couples out a refracted beam that is then projected against the wall or
 1052 some ruler at large distance to triangulate θ . (D), the positional information contained in the
 1053 reflected beam is used to measure $r(\theta)$ by coupling out a small percentage of the reflected intensity
 1054 with a miniature semitransparent micromirror (μ) in the periphery of the objective and direct it onto
 1055 a position-sensitive detector (PSD), e.g., a quadrant photodiode.

1056
 1057 FIGURE 5. *Intensity-based techniques for calibrating axial intensity decays*. (A), 'raisin cake', sub-
 1058 diffraction fluorescent microspheres are embedded in an index-matched ($RI \approx n_1$) agarose gel. (B),
 1059 oblique-fluorescent-layer sample, consisting of a fluorophore-coated coverslip and a spacer (of
 1060 height d , typically another cover glass). In a variant, (C), the surface of a long- f lens of known
 1061 curvature radius is coated with fluorescent beads and positioned on the interface. Again the known
 1062 axial fluorophore profile is used to probe the EW decay. In the 'infinitely' thick ($d \gg \lambda$) dye layer
 1063 approach (D), a homogenous fluorophore solution is used to measure the *cumulative* fluorescence at
 1064 a given penetration depth, $\delta(\theta)$. Upon multi- θ sweeps, depending on how far the EW reaches in the
 1065 fluorescent solution, the intensity changes in a predictable manner. (E), in a dilute dye solution, the
 1066 intensity fluctuations resulting from the diffusion of single molecules through the EW-excited
 1067 volume allow measuring the penetration depth through TIR-FCS. (F), a point emitter attached to the
 1068 tip of an atomic force microscope (not shown) or attached to DNA samples the EW in a single
 1069 point.

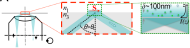
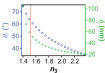
1070
 1071 FIGURE 6. Refractive-index-matched polymer height steps. (A), non-fluorescent polymer
 1072 staircases, onto which sub-diffraction beads are drop cast to produce emitter layers at discrete

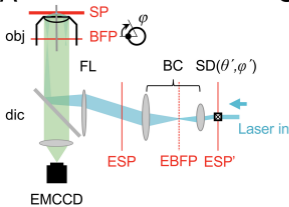
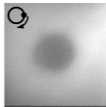
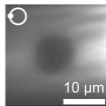
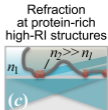
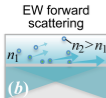
1073 distances, *left*, or covered with a dilute dye solution allow a tomographic reconstruction similar to
 1074 that in Fig. 5D, *right*. (B), multi-layer sandwich of cell index-matched polymer spacer and capping
 1075 layers (grey), separated by a thin fluorophore layer (green). (C), upon EW illumination at an angle
 1076 θ , samples with different fluorophore heights Δ (*top row*) result in different fluorescence intensities
 1077 (*middle*), allowing the measurement of the axial intensity decay (*bottom*) by integrating the total
 1078 fluorescence (*dashed circle*). Note the excitation spots at 3- and 9-o'clock positions on the BFP
 1079 images. The fluorophore distance and refractive index n_1 of the polymer layers are obtained by SAF.
 1080 RI from the radius at which the transition from under- to supercritical angle fluorescence occurs,
 1081 [14]. The SAF/UAF ratio is directly proportional to D , [102].

1082
 1083 FIGURE 7. *Determining the effective penetration depth with a 'raisin-cake' test sample.*
 1084 (A), experimental workflow. *Left*, acquisition of a z -stack of images in epifluorescence (EPI, $\theta =$
 1085 0°). *Middle*, EPI image taken at z_0 ($z := 0$, *red*), localizing the bottom layer of beads on the cover
 1086 slip. *Right*, corresponding TIRF image at z_0 and different beam angles, θ . (B), each bead was
 1087 localized from its axial intensity profile by fitting a Gaussian (*black line*) with the average
 1088 fluorescence, $F(z)$, measured in a 3×3 px ROI (+). *Left*, example bead at $z = 38$ nm, with $F(z)$ well
 1089 described by a Gaussian. *Top* images correspond to planes identified by arrows. *Right*, example of a
 1090 distorted profile discarded from analysis, $z = 44$ nm. (C), Intrinsic fluorescence, i.e., bead intensity
 1091 measured from the peak of the Gaussian as in (B) for each bead, vs. its in-focus position z . (D), bead
 1092 fluorescence $F^{(\text{EPI})}(z; z_0)$ when focusing at the lowest bead layer at z_0 , upon EPI illumination, as a
 1093 function of previously measured bead position z and after normalization for its respective intrinsic
 1094 fluorescence as in (C). The observed axial intensity decay (exponential fit, $D_z = 481 \pm 166$ nm) is the
 1095 result of the increasing defocus for surface-distant beads and the objective's finite depth of field and
 1096 distance-dependent collection efficiency. (D), same, for TIRF excitation (\bullet) and fit of a double-
 1097 exponential decay (*line*) with the measured fluorescence $F^{(\text{TIRF})}(z; z_0)$ from all beads. Color codes for
 1098 different θ . The long-range component (D_z) was identical to that measured for out-of-focus beads
 1099 upon EPI excitation, (D), comforting our interpretation of spurious non-evanescent excitation. The
 1100 short-range component $\delta(\theta)$ was taken as the effective EW the penetration depth and was,
 1101 respectively, 349 ± 118 , 139 ± 20 , 109 ± 16 and 91 ± 13 nm for $\theta = 65.0^\circ$, 67.5° , 70.0° , and 72.5° . Note
 1102 the surface-enhancement by a factor of ~ 4 of $F^{(\text{TIRF})}(z_0)$ vs. $F^{(\text{EPI})}(z_0)$, as expected from theory.
 1103 Depending on θ , the fractional amplitude of the non-evanescent long-range excitation component
 1104 varied between 13 and 17%, see main text. *Inset* shows double-exponential fits on a log scale and
 1105 95% confidence interval of the fit (*thin lines* and *shaded* in the respective color code). In all cases,
 1106 the measured short-range component $\delta(\theta)$ was larger than predicted by theory.

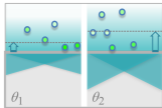
1107
 1108 FIGURE S1. *Method for fixing beads on and above a glass coverslip.* Prior to the entire procedure a
 1109 dash with a permanent marker pen was made on the upper face of the coverslip to facilitate focusing
 1110 at the reflecting interface. Then, *left*, 1 μl of a 1:5 diluted solution of 0.1- μm diameter TetraSpeckTM
 1111 was pipetted to the glass coverslip. Beads were left to dry so that they adhered to the glass.
 1112 Subsequently, *middle*, 20 μl of agarose/sucrose solution again containing TetraSpeckTM beads at a
 1113 1:4 dilution was applied to the coverslip. The agarose was allowed to cool for polymerization. *Right*,
 1114 the whole was topped with ~ 1.5 ml agarose/sucrose ($n_3 = 1.375$) solution previously kept at 35°C .

1115 After 10 min at 4°C the entire solution jellified. The result is a low-density carpet of beads attached
1116 to the coverslip, super-seeded with beads at different heights above the coverslip.

A**B****C**

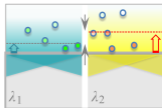
A**C****B**

A



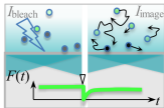
$$\delta \propto (\sin \theta)^{-1}$$

B

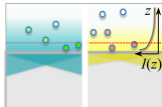


$$\delta \propto \lambda$$

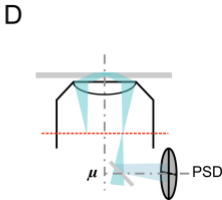
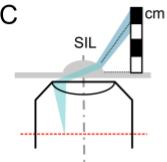
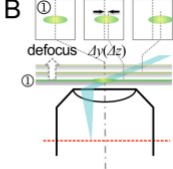
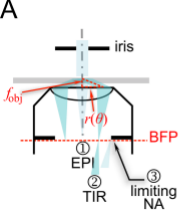
C



FRAP



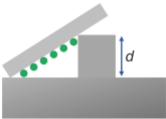
$$\delta(\theta; \lambda)$$



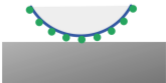
A



B



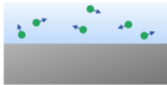
C



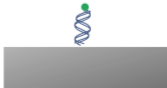
D

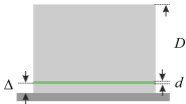


E



F



A**B****C**