



**HAL**  
open science

# Supercritical Angle Fluorescence Microscopy and Spectroscopy

Martin Oheim, Adi Salomon, Maia Brunstein

► **To cite this version:**

Martin Oheim, Adi Salomon, Maia Brunstein. Supercritical Angle Fluorescence Microscopy and Spectroscopy. *Biophysical Journal*, 2020, 118 (10), pp.2339-2348. 10.1016/j.bpj.2020.03.029 . hal-03103103

**HAL Id: hal-03103103**

**<https://cnrs.hal.science/hal-03103103v1>**

Submitted on 20 May 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 *Biophysical Review*

2

3 **Supercritical angle fluorescence microscopy and spectroscopy**

4

5

6 *Running title:* Fluorescence imaging from forbidden angles

7

8

9

10

11 Martin Oheim,<sup>\*,a,✉</sup> Adi Salomon,<sup>\*,†,b</sup> and Maia Brunstein,<sup>\*,‡</sup>

12

13

14 \*Université de Paris, SPPIN - Saints-Pères Paris Institute for the Neurosciences, CNRS, Paris  
15 F-75006, France;

16 †Institute of Nanotechnology and Advanced Materials (BINA), Department of Chemistry,  
17 Bar-Ilan University, Ramat-Gan, 52900 Israel;

18 ‡Chaire d'Excellence Junior, Université Sorbonne Paris Cité, Paris, F-75006 France.

19

20

21

22

23

24

25 Address all correspondence to

26

27

28 ✉ martin.oheim@parisdescartes.fr

29

30 SPPIN - Saints-Pères Paris Institute for the Neurosciences  
31 Centre National de la Recherche Scientifique, CNRS UMR 8003  
32 Université de Paris, Campus Saint-Germain-des-Prés  
33 45 rue des Saints Pères  
34 F-75006 Paris, France

35

36 Phone: +33 1 42 86 42 21 (lab), -42 22 (office)

37

38

39

40

41

42

43 <sup>a)</sup> MO was a *Joseph Meyerhof Invited Professor* with the Department of Biomolecular  
44 Sciences, The Weizmann Institute for Science, Rehovot, Israel, during the academic  
45 year 2018-19.

46

47 <sup>b)</sup> Adi Salomon is currently a *Chateaubriand Senior Research Fellow* with the SPPIN.

48 ABSTRACT. Fluorescence detection, either involving propagating or near-field emission, is  
49 widely being used in spectroscopy, sensing and microscopy. Total internal reflection  
50 fluorescence (TIRF) confines fluorescence excitation by an evanescent (near-) field and it is a  
51 popular contrast generator for surface-selective fluorescence assays. Its emission equivalent,  
52 supercritical angle fluorescence (SAF) is comparably less established although it achieves a  
53 similar optical sectioning as does TIRF. SAF emerges when a fluorescing molecule is located  
54 very close to an interface and its near-field emission couples to the higher-refractive index  
55 medium ( $n_2 > n_1$ ) and becomes propagative. Then, most fluorescence is detectable on the side  
56 of the higher-index substrate and a large fraction of this fluorescence is emitted into angles  
57 forbidden by Snell's law. SAF as well as the undercritical angle fluorescence (UAF, far-field  
58 emission) components can be collected with microscope objectives having a high-enough  
59 detection aperture ( $NA > n_2$ ) and be separated in the back-focal plane (BFP) by Fourier  
60 filtering. The BFP image encodes information about the fluorophore radiation pattern, and it  
61 can be analysed to yield precise information about the refractive index in which the emitters  
62 are embedded, their nanometric distance from the interface and their orientation. A SAF  
63 microscope can retrieve this near-field information through wide-field optics in a spatially  
64 resolved manner, and this functionality can be added to an existing inverted  
65 microscope. Here, we describe the potential underpinning of SAF microscopy and spectro-  
66 scopy, particularly in comparison with TIRF. We review the challenges and opportunities that  
67 SAF presents from a biophysical perspective, and we discuss areas where we see potential.

68 [220 words]

69

## 70 **Significance statement**

71 Many processes happen close to or at an interface. Battery electrodes, biofilm growth,  
72 transport and signaling across cellular membranes, or phase mixing are just a few examples of  
73 such phenomena. While fluorescence-based detection techniques are standard, their surface-  
74 selective variants require the elimination of bulk background fluorescence to detect near-  
75 interface molecules in isolation. Near-field techniques usually require specialized instrument-  
76 tations but there is an optical phenomenon called supercritical-angle fluorescence (SAF) that  
77 makes near-field information available in the far field. As SAF can originate from near-  
78 interface fluorophores only and these molecules emit a highly directional fluorescence, SAF  
79 has a unique signature that permits its separation from classical bulk fluorescence. SAF can  
80 be captured by a high-numerical aperture objective and imaged in its back-focal plane and it  
81 contains not only information about the molecule, but also about its environment. [997 charac.]

## 82 INTRODUCTION

83  
84 SUPERCRITICAL ANGLE FLUORESCENCE<sup>1</sup> (SAF) designates fluorescence directed into emission  
85 angles forbidden for far-field light. Virtually unknown even among experts only a couple of years ago,  
86 SAF is increasingly being used in, fluorescence detection and spectroscopy, but also in near-surface  
87 imaging applications and in SAF-based refractometry. A growing body of super-resolution literature  
88 combines SAF for nanometer axial fluorophore localization (along the microscope's optical axis) with  
89 other techniques that improve the lateral resolution. This *Biophysical Review* provides an update on  
90 the growing field of SAF and it is organized in five sections. In the first section, we review the physics  
91 of dipole near-field emission and explain how SAF becomes detectable in the far-field. We argue that  
92 SAF can be understood as the optical reciprocal of TIRF with a similar optical section capacity. In  
93 section II, we discuss the use of SAF for imaging applications, and we also explore the (at first sight  
94 not-so-obvious) interest of combined TIRF excitation and SAF detection. In the third chapter, we shed  
95 light on the different experimental strategies to separate under- and supercritical emission components,  
96 before section IV gives an overview of recent SAF applications. In the final section V, we try to  
97 anticipate future directions of research and we give a subjective perspective on what might be next in  
98 biophysical uses of SAF.

99

100

## 101 I. SUPERCRITICAL ANGLE FLUORESCENCE, A TWIN AND COMPANION TO TIRF

102

103

104

105

*“If you can see me, I can see you”*

106 OPTICAL RECIPROCITY is key for understanding SAF, or “forbidden-light detection” (1). SAF  
107 has been dubbed “evanescence in emission” (2), much as TOTAL INTERNAL REFLECTION  
108 fluorescence (TIRF) refers to “evanescence in excitation”. Common to either optical pheno-  
109 menon are near-field interactions of light with a dielectric material. When light hits the  
110 interface, the angles of refraction and reflection are governed by SNELL'S LAW, and the  
111 transmitted and refracted intensities are given by the Fresnel coefficients, **Fig. 1A (top)**. For

---

<sup>1</sup> Terms in SMALL CAPS are explained in the Glossary at the end of this Review Article.

112 supercritical angles, i.e.,  $\theta_2 \geq \theta_c = \text{asin}(n_1/n_2)$  the reflection is total so that  $I_{\text{refl}} = I_0$  and an  
 113 evanescent wave (EW) is generated just above the boundary as a consequence of energy and  
 114 momentum conservation. This inhomogeneous surface wave skims the optically rarer medium  
 115 and it propagates along  $+x$  for an incidence from the left as illustrated in panel A. The EW  
 116 intensity decays exponentially with distance  $z$  from the interface with a length-constant  $\delta =$   
 117  $\lambda/[4\pi(n_2^2 \cdot \sin^2(\theta) - n_1^2)^{1/2}]$ . For most realistic optical interfaces  $\delta$  is of the order of  $\lambda/4$ . Here  $\lambda$   
 118 denotes the wave-length of light. If a fluorophore is present at a height  $h$  above the interface,  
 119 then the EW excites fluorescence in proportion to  $I(h)/I_0 \sim \exp[-h/\delta \sin(\theta)]$ , **Fig. 1A (bottom)**.  
 120 Thus, fluorescence excitation will be confined to a thin slice of roughly  $2\delta$  thickness, as  
 121 further discussed in refs. (3, 4).

122 Fluorescence emission has both near- and far-field components. The far-field of a  
 123 radiating dipole displays the well-known ‘laying-eight’ angular emission pattern with an  
 124 intensity maximum perpendicular to the dipole axis, **Fig. 1B (top)**. When the molecule rotates,  
 125 (e.g., in solution) its time-averaged far-field emission will be isotropic. For a dipole located at  
 126 a height  $h \gg \lambda$  from the dielectric interface the collected fraction of isotropically emitted  
 127 fluorescence scales as  $\Omega/4\pi = \Phi = \frac{1}{2} [1 - \cos(\theta_{\text{NA}})] = \frac{1}{2} [1 - (1 - (\text{NA}/n_2)^2)^{1/2}]$ , where  $\theta_{\text{NA}} =$   
 128  $\text{asin}(\text{NA}/n_2)$ . For a NA-1.45 TIRF objective,  $\Phi$  is of the order of 36%, which means that only  
 129 one third of the total emitted fluorescence is collected, and this despite the large NA of the  
 130 TIRF objective lens.

131 In addition to its far-field, dipole emission has a near-field component, too (2). Dipole  
 132 emission in uniform media has components that decay  $\propto 1/r$ ,  $1/r^2$  and  $1/r^3$ . The exponentially  
 133 dwindling ‘evanescent’ component only exists in the presence of an interface between optical  
 134 media with different indices of refraction. ( $h \leq \lambda$ ). The emission is converted by the proximity  
 135 of the dielectric boundary into a propagating wave, similar to the appearance of a propagating  
 136 transmitted wave when a second prism is positioned in close proximity above the reflecting

137 interface in the case of frustrated TIR (5). Analogous to the classical ‘double-prism’  
138 experiment, this propagating wave is directed into angles “forbidden” by Snell’s law, and the  
139 phenomenon is hence termed “supercritical angle fluorescence” (SAF), **Fig. 1B (bottom)** (2, 6,  
140 7). Only fluorophores located very close to the dielectric boundary can emit SAF. The closer  
141 they are, the more of their emission is funneled into supercritical angles making SAF. Its  
142 near-exponential distance dependence makes SAF an absolute nanometric axial ruler  
143 insensitive to most aberration biases (6, 8-10). In turn, fluorophores distant from the interface  
144 cannot emit SAF at all, and their fluorescence is necessarily undercritical, **Fig. 1C**. Thus, the  
145 selective detection of SAF vs. undercritical angle fluorescence (UAF) allows discriminating  
146 near-interface fluorophores against those located in the bulk (1).

147

## 148 II. SELECTIVE SAF DETECTION PERMITS THE ACQUISITION OF TIRF-LIKE IMAGES

149 Equally based on near-field/interface interactions, SAF and TIRF produce a similar optical  
150 sectioning. SAF collection in the solid-angle range above  $\theta_c$  renders fluorescence *detection*  
151 surface-selective, much as supercritical fluorescence *excitation* confines TIRF. In TIRF, light  
152 propagating at high incidence angles produces a near-field close to an interface; in SAF, a  
153 near-field close to an interface generates high-angle emission. This is the optical reciprocity  
154 of TIRF and SAF. The similar axial confinement of both near-field techniques is illustrated in  
155 a cell-biological application by the pseudo-color overlay of TIRF and SAF images of green-  
156 fluorescent protein- (GFP-) expressing mitochondria in cultured cortical astrocytes taken with  
157 the same 1.46-NA objective, **Fig. 2A (i)**. TIRF images were acquired upon azimuthal beam  
158 spinning (11) at polar beam angles of  $\theta = 64, 68$  and  $70^\circ$ , respectively, (*green*), and the SAF  
159 image (shown in *red* pseudocolor) was taken upon EPI-excitation ( $\theta = 0$ ). For TIRF, the  
160 precise incidence angle  $\theta$  determines the EW penetration depth  $\delta(\theta)$  (although the ‘true’  
161 excitation light distribution can be contaminated by unwanted stray light and long-range

162 excitation components (4, 12)). On the contrary, SAF exclusively depends on the axial  
163 fluorophore height  $h$ , so that the apparent overlap between TIRF and SAF images will  
164 generally vary with  $\theta$  (13). Using Pearson's and Manders' coefficients (14) to quantify,  
165 respectively, the amount of co-variance of intensities and the overlap between the 'red' and  
166 'green' pixels, we recognize that TIRF and SAF highlight similar structures ( $R_{12} \approx 0.8$ ) but the  
167 fraction of pixels that overlap on the segmented SAF image with TIRF pixels ( $M_2$ ) is steadily  
168 diminishing for higher  $\theta$ , **Fig. 2B**. This is plausible, because the TIRF excitation volume  
169 shrinks with increasing angle and becomes gradually more selective, eventually leading to a  
170 thinner optical section than SAF at very high beam angles, **Fig. 2A (ii)**.

171         However, grazing beam angles require positioning the focused laser spot very close to  
172 the objective's limiting  $\theta_{NA}$ , which can produce stray excitation and degrade the excitation  
173 confinement and image contrast (12). In objective-type TIRF (15), several studies have  
174 documented the presence of an unwanted 5-10% of non-evanescent (long-range) excitation  
175 component that adds to the localized EW excitation (4, 12, 16, 17). Advantageously, TIRF  
176 excitation and SAF detection can be associated to relieve constraints on  $\theta$  (13). Due to the  
177 combined excitation confinement and emission axial filtering, TIR-SAF images display a  
178 better optical sectioning than TIRF alone at the same  $\theta$ , as result of suppressing contribution  
179 of the unwanted fluorescence from fluorophores located at deeper sample regions. Yet, as  
180 only the supercritical part of dipole radiation is being used, SAF images tend to be dimmer.  
181 Nevertheless, as a consequence of the efficient background suppression, they have a better  
182 signal-to-background ratio (SBR) than TIRF-alone images for the same excitation intensities  
183 as seen in a proof-of-concept experiment using tiny fluorescent microspheres as a  
184 fluorescence standard, **Fig. 2C**.

185         Of course, SAF does not require TIRF excitation and SAF detection has been  
186 combined with many other excitation geometries, including epi-fluorescence, confocal-spot

187 excitation without (1, 18, 19) and with stimulated-emission depletion (STED) (20). A  
 188 growing body of spot-excitation and confocal SAF detection is devoted to supercritical  
 189 fluorescence correlation spectroscopy (FCS) (21-24). Finally, dipole-surface interactions and  
 190 supercritical-angle detection are not restricted to fluorescence: supercritical-angle  
 191 luminescence (SAL) detection (25) and supercritical-angle RAMAN (SAR) spectroscopy (26)  
 192 have been reported.

193

### 194 III. SELECTIVE DETECTION OF SUPER- CRITICAL EMISSION COMPONENTS

195 Whereas TIRF can be detected without specialized optics<sup>2</sup>, **Fig. 3A**, for SAF to be detected  
 196 one requires, (i), a *detection*  $NA > n_1$  spanning supercritical angles and, (ii), a device for  
 197 angular emission filtering to reject the undercritical emission component that is dominated by  
 198 fluorophores in the bulk volume.

199 In early implementations, a low-NA central detection aperture and a surrounding high-  
 200 NA parabolic reflector were used to separate under- and supercritical emission components  
 201 (1, 18, 22, 34). When using the highly corrected imaging optics of a high-NA microscope  
 202 objective (2, 9, 10, 13), Abbe's sine condition provides an unambiguous relationship between  
 203 emission angles and radii in the objective's backfocal plane (BFP), providing a rationale for  
 204 FOURIER-PLANE FILTERING,  $r_\theta = f_{\text{obj}} \cdot n_2 \cdot \sin \theta$ . Here,  $f_{\text{obj}}$  is the objective's focal length,  $f_{\text{TL}}/M$ ,  
 205 i.e., the ratio of the focal length of the manufacturer tube lens and the objective transversal  
 206 magnification; see Ref. (27) for the subtleties for very-high NA objectives. One might  
 207 imagine to block UAF emission by simply placing an appropriately sized opaque disk in the  
 208 BFP. However, the BFP is located inside the objective lens, **Fig. 3B**, so that a relay telescope  
 209 is needed to image the BFP into a CONJUGATE PLANE, **Fig. 3C**. A caveat is that in either of the  
 210 geometries shown in panels *B* and *C* the effective detection aperture is reduced to a ring, so

---

<sup>2</sup> But TIRF needs a high-NA objective ref. (15) or, alternatively, the external prism ('Kretschmann') geometry for guiding light a supercritical angles to the interface.



211 that the lower-NA Fourier components are clipped, and only a tiny part of the objectives  
212 SPATIAL-FREQUENCY BANDWIDTH is used for image formation. However, while the wide-field  
213 image shows a degraded lateral resolution, the point-spread function is not significantly  
214 perturbed for confocal-spot detection (**Fig. 3C**), where a lateral resolution of  $226 \pm 12$  nm vs.  
215  $211 \pm 9$  nm has been reported in the SAF and confocal mode, respectively (18).

216 Thus, the SAF image obtained by just blocking out UAF has a lower resolution than  
217 the corresponding image taken at full aperture (28, 29). The precise positioning of an aperture  
218 stop is facilitated by a BERTRAND LENS, **Fig. 3D**.

219 The spatial-resolution problem is elegantly avoided with *virtual SAF* (vSAF) detection  
220 (30). Here, an iris rather than a central disk is used as an aperture mask, and two images are  
221 acquired: one taken at the full NA, and the other with the effective detection aperture stopped  
222 down to  $NA' \approx RI_{\text{sample}}$  ( $\Leftrightarrow r_c = f_{\text{obj}} \cdot n_1$ , where  $r_c$  is the critical radius marking the transition of  
223 UAF to SAF), **Fig. 3E**. The first image contains both SAF and UAF emission components,  
224 the latter contains UAF only. Their difference image,  $v\text{SAF} = (\text{SAF} + \text{UAF}) - \text{UAF}$ , is a  
225 ‘virtual’ SAF image, equivalent to the one taken with the ring aperture and containing  
226 exclusively the fluorescence components emitted into supercritical angles. However, as two  
227 high-NA images are used for image formation, the lateral resolution is maintained. The  
228 downside of this image-subtraction approach is that by taking the (possibly small) difference  
229 between two (possibly bright) images, the shot noise of the difference on the resulting vSAF  
230 image can be much larger than the shot noise in straight SAF. If, in addition, the excitation is  
231 not TIRF, then a lot of useless fluorescence will be detected on the UAF image, which only  
232 increases the shot noise of the difference. In conclusion, the vSAF method has the advantage  
233 of preserving lateral resolution, but the disadvantage in photon statistics. Combined TIR-SAF  
234 partially circumvents this problem.

235 Alternative geometries do not use commercial objectives at all. As mentioned before,  
236 early SAF work used a custom high-NA parabolic ring-mirror (1) surrounding a central  
237 undercritical detection (and excitation) optical path instead of a high-NA objective. The same  
238 authors later presented a design that integrates separate high-NA and low-NA pathways  
239 within a custom objective lens (22), but this objective does not seem to be commercially  
240 available. Common to both geometries is that they use a point detection scheme and require  
241 sample scanning for SAF-image acquisition (18), similar to the formally equivalent (but  
242 rarely used) confocal spot-scanning TIRF geometry (31-33).

243

#### 244 **IV. SAF APPLICATIONS: MORE THAN SURFACE-SELECTIVE FLUORESCENCE**

245 SAF can be used with simple aperture filtering as a surface-selective fluorescence technique  
246 and as an alternative to TIRF-based detection. However, when a BFP image is captured  
247 instead of (or in addition to) the sample plane image and the radiation pattern is analyzed,  
248 SAF can provide additional information (10). In the following, we present a non-exhaustive  
249 list of mostly recent applications and we discuss particularly, how SAF compares relative to  
250 various TIRF-based techniques, and we advocate quantitative uses of BFP-image information.

251

#### 252 **SAF applications with aperture filtering**

253 *Near-surface fluorescence.* Surface-selective fluorescence detection is straightforward for  
254 non-imaging (sensing) applications (34, 35) or in confocal-spot scanning geometries (1, 18,  
255 22), because no special requirements are imposed on the illumination optical path. This  
256 approach particularly interesting for background rejection either in large-area, multi-spot or  
257 multiplexed (multi-well) assays. Indeed SAF is increasingly being used for surface binding  
258 assays, the characterization of antibodies or sensitive toxin detection. In an attempt to scale up  
259 SAF sensing for even higher throughput, miniaturized parabolic optics have recently been  
260 integrated into the bottom of disposable plastic biochips (36) and polymer test tubes (37). A

261 truncated cone-shape geometry was shown to parallelize sensing in 256 spots on a  
262 microscope-slide sized biochip (38, 39). Yet, while useful for sensing, the large aberrations  
263 and missing central cone in these plastic “SAF chips” are prohibitive for imaging  
264 applications.

265  
266 *A short-sighted microscope.* (a), implemented in a scanning geometry combining confocal-  
267 spot illumination with SAF collection on a point detector (18, 33) SAF is a powerful  
268 alternative to TIRF and related excitation-light confining ‘highly inclined laminar optical  
269 sheet (HILO) techniques. However, while the lateral resolution and axial optical sectioning  
270 can of the order of, respectively, 220 and 100 nm (18) the stage-scanning requirement makes  
271 image acquisition too slow for imaging near-membrane dynamics in live cells.

272 (b), as a full-field technique either with a resolution-compromising aperture disk (29)  
273 or else in the resolution-preserving vSAF variant (13, 30, 40), SAF microscopy is a simple,  
274 cheap and less alignment-demanding whole-field alternative to TIRF. Additionally, no laser  
275 source is needed. Compatible with a host of illumination schemes and readily implemented on  
276 a standard inverted microscope, vSAF imaging is increasingly being used for imaging cell  
277 adhesion, cytoskeletal, vesicle- and membrane dynamics (see, however, the above comment  
278 on shot noise doubling). Also, we observed in vesicle-tracking experiments using FM1-43  
279 labeling that SAF ‘magnified’ the relative importance of spurious, membrane-resident  
280 fluorophores that are more efficiently detected than their vesicular counterparts, only a few  
281 tens of nm deeper in the cell. Likewise, when used with epifluorescence excitation, the  
282 presence bulk fluorophores can dominate over the fainter SAF signal if  $c_{\text{bulk}} \gg c_{\text{membrane}}$ .  
283 Thus, some prudence and careful controls are required for quantitative SAF uses and,  
284 depending on the specific fluorophore concentration profile, selective SAF detection can even  
285 reduce the signal-to-background ratio.

286 We advocated the use of SAF in conjunction with TIRF (13), particularly when using  
287 TIRF in the prismless, objective-type configuration. Here, SAF can help to remove impurities,  
288 imperfections and unknowns associated with the passage of the laser beam close to the  
289 limiting NA of the objective and those resulting from conjugate image planes inside the  
290 microscope body (4, 40). Also, contrary to the earlier discussed case of SAF with epifluo-  
291 rescence illumination, the combined excitation and emission optical sectioning result in a  
292 better surface-selectivity of fluorescence detection. Associated to the structured-illumination  
293 variant of TIRF, TIRF-SIM (41-45) SAF is expected to provide a true isotropic ~100-nm  
294 resolution.

295  
296 *Nanometer-axial fluorophore localization.* The steep dependence of the SAF fraction of  
297 emitted fluorescence on the axial fluorophore distance  $h$  has prompted the use of SAF as a  
298 nanometric axial ruler with single-molecule sensitivity in a 150-200 nm range close to the  
299 interface (7, 10, 22). 3-D single-molecule localization with nm precision is offered through  
300 the combination of dSTORM and SAF (8). Hence, one prominent use of SAF is to remove  
301 the traditional imbalance of lateral and axial resolution in super-resolution microscopies by  
302 the SAF-based axial single-molecule localization.

303 We believe that this use of SAF as nanometric axial ruler is particularly powerful, as the  
304 SAF/UAF ratio (near-) exponentially depends on the axial fluorophore distance, but not on  
305 other (linear) factors, like the local illumination intensity, the fluorophore concentration or  
306 detection efficiency across the field-of-view that all cancel out during ratioing. Obvious  
307 applications include PALM, STORM, STED and TIRF-SIM microscopies with combined  
308 SAF detection.

309

310 *Fluorescence correlation spectroscopy (FCS)*. The combined effect of confocal detection and  
311 SAF-mediated surface confinement can produce a tiny (attoliter,  $10^{-18}$  l) detection volume.  
312 This feature has been used for ultrasensitive near-interface diffusion and concentration  
313 measurements via FCS (21, 24, 46). Simultaneous UAF and SAF detection permits the  
314 synchronous readout of bulk and near-surface mobility and concentration (47) and removes  
315 ambiguities resulting from axial motion in non-flat membrane stretches (23). Unlike TIR-  
316 FCS, SAF – and SAF-UAF-ratioing – has the advantage of being less sensitive to the  
317 excitation-light distribution and to define an observation volume through a unique  
318 fluorophore property, axial distance. A detailed treatment of the observation volume in a  
319 confocal-spot geometry is found in ref. (18).

320

### 321 **SAF applications with BFP imaging and the analysis of the radiation pattern**

322 SAF goes beyond axial optical sectioning when imaging the aperture (pupil) plane of a high-  
323 NA objective rather than its field (sample) plane, **Fig. 3D**. The BFP image contains the  
324 fluorophore radiation pattern, which contains fluorophore information (see (10) for a recent  
325 overview). Depending on the exact geometry used, this information is derived either from a  
326 limited number of fluorophores in the confocal spot (33) or else integrated (averaged) over the  
327 entire field of view (40). In addition, how typical aberrations of high-NA objectives influence  
328 Fourier-plane images has been studied in detail (27).

329

330 *Imaging molecular orientation*. Albeit not called SAF at the time but “single-molecule  
331 detection with Fourier-plane image analysis”, the dependence of the fluorophore radiation  
332 pattern on the orientation of the dipole axis relative to the optical axis has allowed studying  
333 molecular orientation (48), rotational dynamics (49, 50) and even nanometric axial movement  
334 in single-molecule (9) and single-nanoparticle detection assays (8), using a combination of

335 lateral single-molecule localization and SAF-based axial-distance assays. Backfocal imaging  
336 and spectroscopy have been applied not only to study angular emission patterns of fluorescent  
337 molecules, but also to investigate anisotropic Raman signals from molecules, elastic  
338 scattering, plasmonic scattering and non-linear scattering as well as secondary emission via  
339 optical antennas (see, e.g., (51) for a recent review and literature cited therein).

340  
341 *Variable-angle SAF spectroscopy.* Surface-selective (immuno-) assays often require a wash  
342 step prior to read-out due to the otherwise overwhelming signal of the large number of  
343 unbound (bulk) fluorescent molecules that dominate over the signal from the molecules of  
344 interest, bound to the substrate. TIRF has been the method-of-choice for investigating the  
345 population of surface-bound fluorophores in isolation, and variable-angle scans (VA-TIRF)  
346 have allowed a ‘tomographic’ reconstruction of layer thickness, membrane topography, or  
347 cell adhesion from fluorophore distance (52-58).

348 SAF is an obvious alternative to VA-TIRF, but how the radiation pattern changes in  
349 the presence of surface-bound and bulk fluorophores had not been experimentally studied  
350 (59). The van Dorpe lab recently investigated how the collection angle influences the SAF  
351 surface sensitivity in the presence of bulk fluorophores. For this, two different fluorophores  
352 were used. One was bound to the surface and the other was dissolved in the bulk. Then,  
353 measuring the spectrum at discrete points in the back focal plane (BFP) by scanning a small  
354 optical fibre along the radial direction, the authors quantified the relative contribution of the  
355 two fluorophores using spectral imaging and linear unmixing. While the highest signal-to-  
356 noise ratio was observed in the region just above  $\theta_c$  because of the higher signal intensity, the  
357 highest signal-to-bulk ratio was observed at much larger angles, at values above  $68^\circ$  for a  
358 glass/water interface. Thus, for experiments where bulk exclusion is important, increasing the  
359 NA enhances the surface sensitivity, however, restricting SAF detection to the outermost

360 high-NA rig comes at the cost of a decreased signal intensity. Obviously, this study inspires  
361 combining emission spectral imaging and spectral BFP image analysis for multi-color studies  
362 of the basal cell membrane or the nanoscale 3-D organization, e.g., for understanding the  
363 nano-architecture of cell adhesion sites and the peripheral multilayered cytoskeleton, which –  
364 at present - are typically being studied with variable-angle TIRF. Also, understanding the  
365 angular dependence on the sensitivity of a SAF biosensor will allow tuning the collection  
366 angles towards specific applications and contribute to designing smaller, more efficient  
367 devices.

368  
369 *Verification of high-NA objectives.* When the RI of the fluorophore-embedding medium is  
370 known (as for air, water, or calibrated sucrose<sup>3</sup> or for a glycerol/water mix (60, 61)), the  
371 calibration of BFP radii via  $r_c = f_{\text{obj}} \cdot n_1$  on a BFP image and application of Abbe's sine  
372 condition allows measuring the outer, bounding radius. This measurement directly provides  
373 the effective objective  $\text{NA}_{\text{eff}}$  via  $r_{\text{NA}} = f_{\text{obj}} \cdot n_2 \cdot \sin \theta = f \cdot \text{NA}_{\text{eff}}$ . SAF-based NA measurements are  
374 a simple and precise means to measure large NAs  $> n_2 \cdot \sin \theta_c$  that are otherwise difficult to  
375 measure because of TIR (62). Using this strategy, we found important deviations between the  
376 true and specified NA for some objectives (Fig. S2 in Ref. (12)). A reduced effective NA is  
377 not only a relevant information prior to buying such expensive objective lenses (e.g., when  
378 upgrading from a 1.45 to a 1.49-NA lens), but it is also a factor causing stray excitation in  
379 TIRF experiments where a good optical sectioning, i.e., very high polar beam angles  $\theta$  are  
380 sought for.

381

---

<sup>3</sup> Degrees Brix (°Bx) is the [sugar](#) content of an aqueous solution. One °Bx is 1 g of [sucrose](#) in 100 g of solution and represents the strength of the solution as mass %. The °Bx is traditionally used in the wine, sugar, carbonated beverage, fruit juice, maple syrup and honey industries, but the commercial availability of calibrated sucrose solution has also been exploited in science. See, e.g., Ref. (40).

382 *SAF refractometry*. Conversely, with  $NA_{eff}$  known, an interesting and new SAF application is  
383 near-surface refractometry (40): as the transition of UAF to SAF depends on the sample RI  $n_1$   
384 (but not on  $n_2$ ), the measurement of the “critical radius”  $r_c = f_{obj} \cdot n_1$  on the BFP image allows  
385 for a sensitive measurement of the *local* RI of the fluorophore-embedding medium. The  
386 precision of this measurement depends on the quality of the fit of the BFP image (and hence  
387 on the available signal, pixel size, and camera sensitivity), and typical RI values with  $\pm 0.001$   
388 error are being reached. This property has been used – together with diverse fluorophores that  
389 we subcellularly targeted to different organelles – for intracellular RI-measurements (40). Of  
390 note, even though wide-field SAF refractometry measures the *local* fluorophore environment,  
391 it reports an *average* RI value,  $\langle n_1 \rangle$ , averaged over all SAF-emitting fluorophores in the  
392 field-of-view. In an attempt to achieve spatially resolved RI measurements, a combination of  
393 Bessel-beam-spot excitation with confocal intensity and simultaneous SAF-based RI  
394 detection through the same high-NA objective has been reported in a recent communication  
395 (33).

396

397 *Label-free RI-based SAF assay*. An interesting variant of this refractometric readout is SAF  
398 detection from a fluorophore-coated glass microcapillary (63). Here, the analyte is non-  
399 fluorescent and its presence sensed indirectly via the RI change. This change in the  
400 fluorophore environment (detected as a shift in  $r_c$  and hence  $\langle n_1 \rangle$ ) is induced by a medium  
401 change or - as shown by the authors – by bacterial growth on the capillary bottom. In the  
402 same manner, one can imagine to measure cell adhesion or confluency in unlabeled cell  
403 cultures grown on a fluorophore-coated coverslip. In either case, the sensitivity of this label-  
404 free fluorescent SAF assay will be limited by the fluorescence signal available after  
405 prolonged use of the capillary sensor and by the timecourse of photobleaching of the



406 immobilized dye. Conceptually, the technique is reminiscent of nm-scale axial resolution with  
407 non-radiative excitation, (64).

408  
409 *Incidence-angle calibration in VA-TIRF.* BFP imaging of the SAF-ring has been used for  
410 calibrating the polar beam angle  $\theta$  in objective-type TIRF microscopy. Similar to the earlier  
411 RI-measurement, the approach is based on a series of  $\theta_c$ -measurements on BFP images using  
412 a glass substrate topped with solvents having different RIs. In one study, a thin homogenous  
413 layer of fluorophores in air was used to produce a SAF ring of known radius  $r_c^{(\text{air})} = f_{\text{obj}}$  that  
414 was compared to the annular distribution of excitation light in azimuthal beam-spinning TIRF  
415 with the fluorescence filter removed (65). Two follow-up studies used a similar strategy but  
416 either with a thin layer of organic dye for TIRF-SIM calibration (45) or else with Qdots  
417 embedded in PMMA for A-TIRFM (66). In the latter case, the fluorophore layer was topped  
418 with solutions of different, known RI (previously measured with an Abbe refractometer) and  
419 the laser spot (seen when the emission filter was taken out) steered to these known angles to  
420 attain a more precise multi-angle calibration.

421  
422 **V. WHAT'S NEXT?**  
423 SAF, either used for surface-selective fluorescence detection or – combined with the analysis  
424 of BFP images – for imaging the fluorophore radiation pattern, is an active and rapidly  
425 expanding area of research. Beyond its early uses in spectroscopy, sensing and studies of  
426 single-molecule mobility and rotation, SAF detection plays increasing roles in imaging  
427 applications, either with wide-field detection or else as a confocal point-measurement  
428 technique with sample scanning. Recent studies emphasized the quantitative analysis of BFP  
429 images to extract various fluorophore properties, and ongoing work aims at the real-time  
430 analysis of BFP images through rapid feature extraction. Future studies will likely combine an

431 analysis of the radiation pattern with fluorescence-intensity, life-time or anisotropy-based  
432 contrast, marking the arrival of SAF-based multi-modal microscopies.

433         Given that these applications all rely on BFP image analysis we expect deep-learning  
434 to have an impact similar to other fields of microscopic image analysis. Artificial intelligence  
435 (AI) based BFP image analysis will boost information-extraction from the narrow high-  
436 intensity ring characteristic for SAF. Fast feature recognition – particularly when multiple  
437 emitters or multi-color emitters are present - and adaptive aperture filtering are only two  
438 aspects that will benefit from AI, in a manner similar to exploiting the chromatic dependence  
439 of PSF shapes from different emitters imaged on a grayscale camera (67).

440         The relative ease of use, reduced complexity and (compared to TIRF) low price of  
441 instrumentation required for vSAF is most likely to increase its dissemination of the for  
442 routine biological microscopy. A ‘vSAF-module’ that can be fitted between the microscope  
443 exit port and an existing EMCCD or sCMOS camera is now commercially available (by the  
444 French start-up Abbelight – non-related to the authors), and makes the technique accessible  
445 for non-expert users.

446         Finally, in as much as SAF does not depend on how fluorescence was excited but  
447 represents an intrinsic fluorophore property depending solely on its distance from the  
448 interface, we foresee a growing use of SAF in microscope calibration and standardization  
449 applications (4).

450

451

## 452 **List of abbreviations**

|     |       |   |  |
|-----|-------|---|--|
| 453 | AI    | - | artificial intelligence                    |
| 454 | BFP   | - | back-focal plane                           |
| 455 | EMCCD | - | electron-multiplying charge-coupled device |
| 456 | EW    | - | evanescent wave                            |

|     |        |   |   |
|-----|--------|---|---|
| 457 | FCS    | - | fluorescence correlation spectroscopy       |
| 458 | FWHM   | - | full width at half maximum                  |
| 459 | HILO   | - | highly inclined laminar optical (sheet)     |
| 460 | NA     | - | numerical aperture                          |
| 461 | PCC    | - | Pearson's correlation coefficient           |
| 462 | PMMA   | - | poly(methyl methacrylate)                   |
| 463 | PSF    | - | point spread function                       |
| 464 | RI     | - | refractive index                            |
| 465 | SAF    | - | supercritical angle fluorescence            |
| 466 | SBR    | - | signal-to-background ratio                  |
| 467 | sCMOS  | - | scientific complementary metal oxide sensor |
| 468 | STED   | - | stimulated emission depletion microscopy    |
| 469 | TIR(F) | - | total internal reflection (fluorescence)    |
| 470 | UAF    | - | under-critical angle fluorescence           |

471

472

473 **Glossary**

474 APERTURE (OR BACKFOCAL) PLANE – in a multi-lens system, a plane conjugate to the aperture,  
 475 in a light microscope, the set of conjugate aperture planes comprises the exit pupil, objective  
 476 back-focal plane, condenser front focal plane and the lamp filament. (see also field plane for  
 477 the other set of conjugate planes including the sample plane and detector).

478 BERTRAND LENS – an optical device used in aligning the various optical components of a light  
 479 microscope. It allows observation of the back focal plane of the objective lens and its  
 480 conjugated focal planes.

481

482 FOURIER FILTERING – refers to modulating or clipping in frequency space; in SAF, it refers to  
 483 shaping the SPATIAL FREQUENCY BANDWIDTH of the microscope by selectively attenuating,

484 blocking or enhancing certain segments of the (conjugate) back-focal plane by an aperture  
485 mask.

486 NUMERICAL APERTURE ( $NA = n \cdot \sin \theta_{NA}$ ) – Magnitude related to the maximal angle of  
487 collection or excitation of an optical element.

488 (OBJECTIVE) BACK-FOCAL PLANE (BFP) – Effective rear focal plane of the assembly of lens  
489 composing the objective. For most objective lenses, it is located inside the objective and  
490 hence only accessible by generating an intermediate image in a conjugate plane.

491 OPTICAL RECIPROCITY – (for all practical grounds here) principle that describes how a ray of  
492 light and its reverse follow the same optical path.

493 RAMAN (SCATTERING) – Inelastic scattering of a photon when it encounters matter.

494 SNELL'S LAW – A law from geometrical optics that describes how a ray of light is reflected  
495 and/or transmitted when it encounters an interface between two different refractive-index  
496 media,  $n_i \cdot \sin \theta_i = \text{const.}$

497 SPATIAL-FREQUENCY BANDWIDTH - Range of frequencies that is transmitted by the system.  
498 In an optical (or imaging) system this bandwidth is inversely related to the spatial optical  
499 resolution. See also Object transfer function and Point Spread Function.

500 SUPERCRITICAL ANGLE FLUORESCENCE (SAF) – Fluorescence that propagates through a higher-  
501 index medium (compared to the emission medium) at 'forbidden' angles higher than the  
502 critical angle.

503 TOTAL INTERNAL REFLECTION (TIR) – Complete bouncing back of a beam from a transparent  
504 interface (i.e., no transmitted light) that can occur when a light impinges at an interface from  
505 the higher- ( $n_2$ ) to the lower-refractive index side ( $n_1$ ), at an angle larger than a certain critical  
506 angle,  $\theta_c = \text{asin}(n_2/n_1)$ .

507

508 ACKNOWLEDGEMENTS

509 This study was supported by a *Chaire d'Excellence Junior University Sorbonne Paris Cité*,  
510 USPC (to MB), the Israeli Science Foundation (ISF-NSFC 2525/17, to AS), the European  
511 Union (H2020 Eureka! Eurostars NANOSCALE (to MO and AS). Our collaborative research  
512 on TIRF and SAF was further financed by a French-Israeli ImagiNano CNRS-LIA grant (to  
513 AS & MO), the *Agence Nationale de la Recherche* (ANR-10-INSB-04-01, *grands*  
514 *investissements* FranceBioImaging, FBI, to MO), the Université Paris Descartes (invited  
515 professorship during the academic year 2017-18, to AS) and the French *Ministère des Affaires*  
516 *Etrangères* (Chateaubriand Senior Fellowship to AS). The authors thank Dana Khanafer for  
517 help with Fig. 2C. The Oheim lab is a member of the C'nano IdF and *Ecole de Neurosciences*  
518 *de Paris* (ENP) excellence clusters for nanobiotechnology and neurosciences, respectively.

519

## 520 **Author contributions**

521 All authors contributed to experiments. The manuscript was written by MO with contributions  
522 of all authors. All authors have given their approval to the final version of the manuscript.

523

## 524 **Notes**

525 The authors declare no competing financial interest.

526

527

## 528 **REFERENCES**

- 529 1. Ruckstuhl, T., J. Enderlein, S. Jung, and S. Seeger. 2000. Forbidden light detection from  
530 single molecules. *Anal. Chem.* 72:2117-2123.
- 531 2. Axelrod, D. 2013. Evanescent excitation and emission in fluorescence microscopy. *Biophys.*  
532 *J.* 104(7):1401-1409.
- 533 3. Oheim, M. 2016. TIRF (Total Internal Reflection Fluorescence). eLS. John Wiley & Sons,  
534 Ltd.
- 535 4. Oheim, M., A. Salomon, A. Weissman, M. Brunstein, and U. Becherer. 2019. Calibrating  
536 evanescent-wave penetration depths for biological TIRF microscopy. *Biophys. J.* 117(5):795-  
537 809.
- 538 5. Harrick, N., and K. Beckmann. 1974. Internal reflection spectroscopy. Characterization of  
539 Solid Surfaces. Springer, pp. 215-245.

- 540 6. Hellen, E. H., and D. Axelrod. 1987. Fluorescence emission at dielectric and metal-film  
541 interfaces. *JOSA B* 4(3):337-350.
- 542 7. Axelrod, D. 2012. Fluorescence excitation and imaging of single molecules near dielectric-  
543 coated and bare surfaces: a theoretical study. *J. Microsc.* 247(2):147-160.
- 544 8. Bon, P., N. Bourg, S. Lécart, S. Monneret, E. Fort, J. Wenger, and S. Lévêque-Fort. 2015.  
545 Three-dimensional nanometre localization of nanoparticles to enhance super-resolution  
546 microscopy. *Nat. Commun.* 6:7764.
- 547 9. Bourg, N., C. Mayet, G. Dupuis, T. Barroca, P. Bon, S. Lécart, E. Fort, and S. Lévêque-Fort.  
548 2015. Direct optical nanoscopy with axially localized detection. *Nat. Photonics* 9(9):587.
- 549 10. Brunstein, M., A. Salomon, and M. Oheim. 2018. Decoding the information contained in  
550 fluorophore radiation patterns. *ACS Nano* 12(12):11725-11730.
- 551 11. van't Hoff, M., V. de Sars, and M. Oheim. 2008. A programmable light engine for quantitative  
552 single molecule TIRF and HILO imaging. *Opt. Express* 16(22):18495-18504.
- 553 12. Brunstein, M., M. Teremetz, K. Hérault, C. Tourain, and M. Oheim. 2014. Eliminating  
554 unwanted far-field excitation in objective-type TIRF. Part I. identifying sources of  
555 nonevanescent excitation light. *Biophys. J.* 106(5):1020-1032.
- 556 13. Brunstein, M., K. Hérault, and M. Oheim. 2014. Eliminating unwanted far-field excitation in  
557 objective-type TIRF. Part II. combined evanescent-wave excitation and supercritical-angle  
558 fluorescence detection improves optical sectioning. *Biophys. J.* 106(5):1044-1056.
- 559 14. Manders, E., F. Verbeek, and J. Aten. 1993. Measurement of co-localization of objects in  
560 dual-colour confocal images. *Journal of microscopy* 169(3):375-382.
- 561 15. Stout, A. L., and D. Axelrod. 1989. Evanescent field excitation of fluorescence by epi-  
562 illumination microscopy. *Applied optics* 28(24):5237-5242.
- 563 16. Mattheyses, A. L., and D. Axelrod. 2006. Direct measurement of the evanescent field profile  
564 produced by objective-based total internal reflection fluorescence. *J. Biomed. Opt.*  
565 11(1):014006-014006-014007.
- 566 17. Niederauer, C., P. Blumhardt, J. Mücksch, M. Heymann, A. Lambacher, and P. Schwille.  
567 2018. Direct characterization of the evanescent field in objective-type total internal reflection  
568 fluorescence microscopy. *Opt. Express* 26(16):20492-20506.
- 569 18. Ruckstuhl, T., and D. Verdes. 2004. Supercritical angle fluorescence (SAF) Microscopy. *Opt.*  
570 *Express* 12(18):4246-4254.
- 571 19. Sivankutty, S., T. Barroca, C. Mayet, G. Dupuis, E. Fort, and S. Lévêque-Fort. 2014. Confocal  
572 supercritical angle microscopy for cell membrane imaging. *Opt. Lett.* 39(3):555-558.
- 573 20. Sivankutty, S., I. C. Hernández, N. Bourg, G. Dupuis, and S. Lévêque-Fort. 2019.  
574 Supercritical angle fluorescence for enhanced axial sectioning in STED microscopy. *Methods.*
- 575 21. Ries, J., T. Ruckstuhl, D. Verdes, and P. Schwille. 2008. Supercritical angle fluorescence  
576 correlation spectroscopy. *Biophys. J.* 94(1):221-229.
- 577 22. Ruckstuhl, T., D. Verdes, C. M. Winterflood, and S. Seeger. 2011. Simultaneous near-field  
578 and far-field fluorescence microscopy of single molecules. *Opt. Express* 19(7):6836-6844.
- 579 23. Winterflood, C. M., T. Ruckstuhl, N. P. Reynolds, and S. Seeger. 2012. Tackling Sample-  
580 Related Artifacts in Membrane FCS Using Parallel SAF and UAF Detection. *ChemPhysChem*  
581 13(16):3655-3660.
- 582 24. Ma, Y., A. Benda, P. R. Nicovich, and K. Gaus. 2016. Measuring membrane association and  
583 protein diffusion within membranes with supercritical angle fluorescence microscopy.  
584 *Biomedical optics express* 7(4):1561-1576.
- 585 25. Blue, R., N. Kent, L. Polerecky, H. McEvoy, D. Gray, and B. MacCraith. 2005. Platform for  
586 enhanced detection efficiency in luminescence-based sensors. *Elec. Lett.* 41(12):682-684.
- 587 26. Serrano, D., and S. Seeger. 2017. Supercritical angle Raman microscopy: a surface-sensitive  
588 nanoscale technique without field enhancement. *Light: Sci. & Appl.* 6(10):e17066.
- 589 27. Kurvits, J. A., M. Jiang, and R. Zia. 2015. Comparative analysis of imaging configurations  
590 and objectives for Fourier microscopy. *JOSA A* 32(11):2082-2092.
- 591 28. Enderlein, J., I. Gregor, and T. Ruckstuhl. 2011. Imaging properties of supercritical angle  
592 fluorescence optics. *Opt. Express* 19(9):8011-8018.
- 593 29. Barroca, T., K. Balaa, J. Delahaye, S. Lévêque-Fort, and E. Fort. 2011. Full-field supercritical  
594 angle fluorescence microscopy for live cell imaging. *Optics letters* 36(16):3051-3053.

- 595 30. Barroca, T., K. Balaa, S. Lévêque-Fort, and E. Fort. 2012. Full-field near-field optical  
596 microscope for cell imaging. *Phys. Rev. Lett.* 108(21):218101.
- 597 31. Chon, J. W., and M. Gu. 2004. Scanning total internal reflection fluorescence microscopy  
598 under one-photon and two-photon excitation: image formation. *Appl. Opt.* 43(5):1063-1071.
- 599 32. Terakado, G., K. Watanabe, and H. Kano. 2009. Scanning confocal total internal reflection  
600 fluorescence microscopy by using radial polarization in the illumination system. *Appl. Opt.*  
601 48(6):1114-1118.
- 602 33. Szederkenyi, K., B. Lagarde, M. Brunstein, M. Guillon, C. Yip, and M. Oheim. 2020.  
603 Investigating Polarisation Effects in a Confocal Total Internal Reflection-Supercritical Angle  
604 Fluorescence (TIR-SAF) Geometry with Sample Scanning *Biophys. J.* abstract.
- 605 34. Ruckstuhl, T., M. Rankl, and S. Seeger. 2003. Highly sensitive biosensing using a  
606 supercritical angle fluorescence (SAF) instrument. *Biosens. Bioelec.* 18(9):1193-1199.
- 607 35. Kurzbuch, D., J. Bakker, J. Melin, C. Jönsson, T. Ruckstuhl, and B. MacCraith. 2009. A  
608 biochip reader using super critical angle fluorescence. *Sens. Actuat. B: Chemical* 137(1):1-6.
- 609 36. Hill, D., B. McDonnell, S. Hearty, L. Basabe-Desmonts, R. Blue, M. Trnavsky, C.  
610 McAtamney, R. O'Kennedy, and B. D. MacCraith. 2011. Novel disposable biochip platform  
611 employing supercritical angle fluorescence for enhanced fluorescence collection. *Biomed.*  
612 *microdev.* 13(4):759-767.
- 613 37. Ruckstuhl, T., C. M. Winterflood, and S. Seeger. 2011. Supercritical angle fluorescence  
614 immunoassay platform. *Anal. Chem.* 83(6):2345-2350.
- 615 38. Hung, T. Q., Y. Sun, C. E. Poulsen, T. Linh-Quyen, W. H. Chin, D. D. Bang, and A. Wolff.  
616 2015. Miniaturization of a micro-optics array for highly sensitive and parallel detection on an  
617 injection moulded lab-on-a-chip. *Lab on a Chip* 15(11):2445-2451.
- 618 39. Nguyen, T., T. A. Ngo, D. D. Bang, and A. Wolff. 2019. Optimising the supercritical angle  
619 fluorescence structures in polymer microfluidic biochips for highly sensitive pathogen  
620 detection: a case study on *Escherichia coli*. *Lab on a Chip* 19(22):3825-3833.
- 621 40. Brunstein, M., L. Roy, and M. Oheim. 2017. Near-membrane refractometry using supercritical  
622 angle fluorescence. *Biophys. J.* 112(9):1940-1948.
- 623 41. Fiolka, R., M. Beck, and A. Stemmer. 2008. Structured illumination in total internal reflection  
624 fluorescence microscopy using a spatial light modulator. *Opt. Lett.* 33(14):1629-1631.
- 625 42. Kner, P., B. B. Chhun, E. R. Griffis, L. Winoto, and M. G. Gustafsson. 2009. Super-resolution  
626 video microscopy of live cells by structured illumination. *Nat. Methods* 6(5):339.
- 627 43. Brunstein, M., K. Wicker, K. Hérault, R. Heintzmann, and M. Oheim. 2013. Full-field dual-  
628 color 100-nm super-resolution imaging reveals organization and dynamics of mitochondrial  
629 and ER networks. *Opt. Express* 21(22):26162-26173.
- 630 44. Young, L. J., F. Ströhl, and C. F. Kaminski. 2016. A guide to structured illumination TIRF  
631 microscopy at high speed with multiple colors. *JoVE (J. Vis. Exp.)*(111):e53988.
- 632 45. Chen, Y., R. Cao, W. Liu, D. Zhu, Z. Zhang, C. Kuang, and X. Liu. 2018. Widefield and total  
633 internal reflection fluorescent structured illumination microscopy with scanning galvo mirrors.  
634 *J. Biomed. Opt.* 23(4):046007.
- 635 46. Ries, J., and P. Schwille. 2008. New concepts for fluorescence correlation spectroscopy on  
636 membranes. *Phys. Chem. Chem. Phys.* 10(24):3487-3497.
- 637 47. Winterflood, C. M., and S. Seeger. 2016. Simultaneous surface-near and solution fluorescence  
638 correlation spectroscopy. *J. Fluoresc.* 26(3):753-756.
- 639 48. Mattheyses, A. L., and D. Axelrod. 2005. Fluorescence emission patterns near glass and  
640 metal-coated surfaces investigated with back focal plane imaging. *J. Biomed. Opt.*  
641 10(5):054007.
- 642 49. Lieb, M. A., J. M. Zavislan, and L. Novotny. 2004. Single-molecule orientations determined  
643 by direct emission pattern imaging. *JOSA B* 21(6):1210-1215.
- 644 50. Backer, A. S., and W. Moerner. 2014. Extending single-molecule microscopy using optical  
645 Fourier processing. *J. Phys. Chem. B* 118(28):8313-8329.
- 646 51. Vasista, A. B., D. K. Sharma, and G. P. Kumar. 2018. Fourier plane optical microscopy and  
647 spectroscopy. *digital Encyclopedia of Applied Physics*:1-14.

- 648 52. Reichert, W., P. Suci, J. Ives, and J. Andrade. 1987. Evanescent detection of adsorbed protein  
649 concentration-distance profiles: fit of simple models to variable-angle total internal reflection  
650 fluorescence data. *Appl. Spectrosc.* 41(3):503-508.
- 651 53. Burmeister, J. S., G. A. Truskey, and W. M. Reichert. 1994. Quantitative analysis of variable-  
652 angle total internal reflection fluorescence microscopy (VA-TIRFM) of cell/substrate contacts.  
653 *J. Microsc.* 173(1):39-51.
- 654 54. Oheim, M., D. Loerke, B. Preitz, and W. Stuhmer. 1999. Simple optical configuration for  
655 depth-resolved imaging using variable-angle evanescent-wave microscopy. In *Optical biopsies  
656 and microscopic techniques III*. SPIE, editor. Proc. SPIE - International Society for Optics and  
657 Photonics. 131-140.
- 658 55. Loerke, D., B. Preitz, W. Stuhmer, and M. Oheim. 2000. Super-resolution measurements with  
659 evanescent-wave fluorescence-excitation using variable beam incidence. *J. Biomed. Opt.*  
660 5(1):23-31.
- 661 56. Stock, K., R. Sailer, W. S. Strauss, M. Lyttek, R. Steiner, and H. Schneckenburger. 2003.  
662 Variable-angle total internal reflection fluorescence microscopy (VA-TIRFM): realization and  
663 application of a compact illumination device. *J. Microsc.* 211(1):19-29.
- 664 57. Dos Santos, M. C., R. D eturche, C. V ezy, and R. Jaffiol. 2014. Axial nanoscale localization  
665 by normalized total internal reflection fluorescence microscopy. *Opt. Lett.* 39(4):869-872.
- 666 58. Dos Santos, M. C., R. D eturche, C. V ezy, and R. Jaffiol. 2016. Topography of Cells Revealed  
667 by Variable-Angle Total Internal Reflection Fluorescence Microscopy. *Biophys. J.*  
668 111(6):1316-1327.
- 669 59. James Shirley, F., P. Neutens, R. Vos, M. Mahmud-Ul-Hasan, L. Lagae, N. Verellen, and P.  
670 Van Dorpe. 2018. Supercritical Angle Fluorescence Characterization Using Spatially  
671 Resolved Fourier Plane Spectroscopy. *Anal. Chem.* 90(7):4263-4267.
- 672 60. Hoyt, L. 1934. New table of the refractive index of pure glycerol at 20 C. *Ind. Engin. Chem.*  
673 26(3):329-332.
- 674 61. Vural, U. S., V. Muradoglu, and S. Vural. 2011. Excess molar volumes, and refractive index  
675 of binary mixtures of glycerol+ methanol and glycerol+ water at 298.15 K and 303.15 K. *Bull.*  
676 *Chem. Soc. Ethiopia* 25(1):111-118.
- 677 62. Dai, L., I. Gregor, I. von der Hocht, T. Ruckstuhl, and J. Enderlein. 2005. Measuring large  
678 numerical apertures by imaging the angular distribution of radiation of fluorescing molecules.  
679 *Opt. Express* 13(23):9409-9414.
- 680 63. Ferdman, B., L. E. Weiss, O. Alalouf, Y. Haimovich, and Y. Shechtman. 2018. Ultrasensitive  
681 Refractometry via Supercritical Angle Fluorescence. *ACS nano* 12(12):11892-11898.
- 682 64. Riachy, L., D. El Arawi, R. Jaffiol, and C. V ezy. 2018. Non Radiative Excitation Fluorescence  
683 Microscopy: A New Method for Studying Membrane Adhesion at the Nanoscale. *Biophys. J.*  
684 114(3):164a-165a.
- 685 65. Soubies, E., S. Schaub, A. Radwanska, E. Van Obberghen-Schilling, L. Blanc-F eraud, and G.  
686 Aubert. 2016. A framework for multi-angle TIRF microscope calibration. In *2016 IEEE 13th  
687 International Symposium on Biomedical Imaging (ISBI)*. IEEE, editor. IEEE, Prague, Czech  
688 Republic. 668-671.
- 689 66. El Arawi, D., M. C. Dos Santos, C. V ezy, and R. Jaffiol. 2019. Incidence angle calibration for  
690 prismless total internal reflection fluorescence microscopy. *Opt. Lett.* 44(7):1710-1713.
- 691 67. Michaeli, T., and Y. Shechtman. 2018. Multicolor localization microscopy by deep learning.  
692 arXiv preprint arXiv:1807.01637.  
693



694 **FIGURE LEGENDS**

695 **FIGURE 1. *Evanescence in excitation and emission.*** (A), *top*, we assume for the purpose of this paper  
 696 a planar dielectric interface separating two homogenous media having different refractive indices  
 697 (RIs),  $n_2 > n_1$ . Snell's law  $n_1 \cdot \sin(\theta_1) = n_2 \cdot \sin(\theta_2)$  governs the angles of refraction at a dielectric  
 698 interface. For a glass/water interface ( $n_2 > n_1$ ), light impinging at the interface from the side of the  
 699 denser medium 2 is totally reflected beyond a critical angle of incidence,  $\theta_c = \text{asin}(n_2/n_1)$ . Total  
 700 reflection creates an inhomogeneous, propagating evanescent wave (EW) right above the dielectric  
 701 boundary in medium 1, *bottom*. **S** is the Pointing vector, indicating the direction of energy flux. (B),  
 702 fluorescence emission of near-interface dipoles. Far-field emission pattern from a radiating dipole  
 703 located far from the interface (fluorophore height  $h \gg \lambda$ ) showing the familiar "laying-8" pattern.  
 704 Molecular rotation (arrow) in solution often results in an isotropic fluorescence emission (dashed  
 705 circle). The collected fraction of fluorescence hence depends on the solid angle captured by the  
 706 NUMERICAL APERTURE (NA) of the objective. On the contrary, for  $h \approx \lambda$ , fluorescent specimen does  
 707 not emit fluorescence isotropically, but approximately 2/3 of the fluorescence is emitted into the  
 708 higher-index medium. Of this emission, the main part is directed to solid angles above the critical  
 709 angle. On the contrary, already for  $h = \lambda/2$ , fluorescence above the critical angle is decreased  
 710 dramatically. Hence, like TIRF, supercritical angle fluorescence (SAF) is suited to discriminate  
 711 between molecules at or near to surfaces and other fluorophores in the bulk. (C), provided the  
 712 detection NA of the collection optics is sufficiently large ( $\text{NA} > n_1$ ), supercritical (SAF) and  
 713 undercritical fluorescence emission components (UAF) can be collected simultaneously and separated  
 714 in the in the objective backfocal plane (BFP). SAF and UAF can be distinguished as they travel at  
 715 different NAs (i.e., radii in the BFP), which can be used for aperture filtering (see main text).

716

717 **FIGURE 2. *SAF detection of TIRF-like images and combined TIR-SAF acquisition with improved***  
 718 *contrast.* (A), TIRF (*green*) and (epi-excited) SAF (*red*) images of EGFP-expressing mitochondria in a  
 719 cultured cortical astrocyte. For TIRF the polar beam angle was  $\theta = 64, 68$  and  $70^\circ$  as indicated. (B),  
 720 Evolution of Manders' co-localization coefficient  $M_1$  for SAF (image 1, *red*) and of Pearson's  
 721 correlation coefficient (PCC, *black*, see Ref. (14)). TIRF and SAF highlight similar structures ( $\langle R_{12} \rangle =$   
 722  $0.79 \pm 0.01$ ). Unlike PCC,  $M_1$  measures the amount of TIRF pixels coinciding with SAF pixels  
 723 following thresholding of each channel. TIRF and SAF images are similar for intermediate beam  
 724 angles (lower EW penetration depths), before TIRF becomes more discriminative at very high  $\theta$ .  
 725 However, this object-based analysis obscures the gain in sensitivity by SAF. (C), Contrast of TIRF-  
 726 alone and combined TIR-SAF images. Images of yellow-green emitting 93-nm diameter beads upon  
 727 488-nm excitation and the FWHM and Michelson contrast ('visibility') calculated as  $C_M = (I_{\max} -$   
 728  $I_{\min}) / (I_{\max} + I_{\min})$ . Pixel size was 103 nm,  $t_{\text{exp}} = 50$  ms. Symbols are measured intensity mean  $\pm$  SD from

729 four independent measurements. Through lines are weighted Gaussian fits. SBR was  $4.92 \pm 0.16$ ,  $C_M$   
730  $= 0.71$  and  $\text{FWHM} = 293 \pm 2$  nm for TIRF alone; vs.  $\text{SBR} = 14.4 \pm 1.1$ ,  $C_M = 0.88$ ,  $\text{FWHM} = 311 \pm 1$   
731 nm for TIR-SAF. Thus, while no large difference was observed between  $\text{FWHM}_S$ , the combined  
732 optical sectioning of TIRF excitation and SAF detection almost tripled contrast ( $\times 2.93$ ).

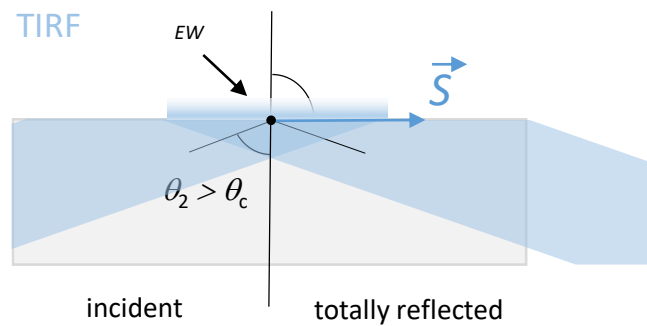
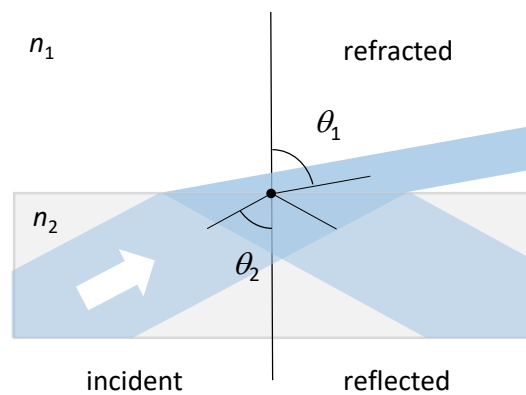
733

734 **FIGURE 3. Microscope geometries for TIRF and SAF detection.** (A), typical optical layout for  
735 prismless ('through-the-objective') type evanescent-wave excitation. In the simplest case, an expanded  
736 laser beam is focused in an eccentric position in the BFP of a high-NA objective. ex – excitation light,  
737 obj – objective lens, dic – dichroic mirror, TL – tube lens, sCMOS – scientific complementary metal-  
738 oxide sensor. (B), hypothetical selective SAF detection with an opaque aperture mask in the objective  
739 BFP. (C), practical implementation of SAF detection with an aperture mask placed in a conjugate  
740 aperture plane, produced by a '4f' relay telescope. Like the arrangement shown in panel C, this  
741 scheme results in a resolution loss due to the ring aperture and clipping of central rays. (D), insertion  
742 of a Bertrand lens (BL) allows BFP (or "Fourier-plane") imaging. (E), virtual SAF, vSAF  
743 configuration, in which an iris stop is used instead of an opaque disk and the SAF image is calculated  
744 from the difference of two images taken with the iris open and stopped down to  $NA' = n_1$ , respectively.  
745 *Inset* images at the bottom represent the fluorescence radiation patterns observed by imaging the BFP  
746 of the objective in the various configurations.

A

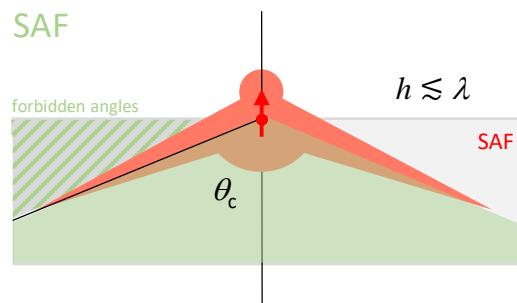
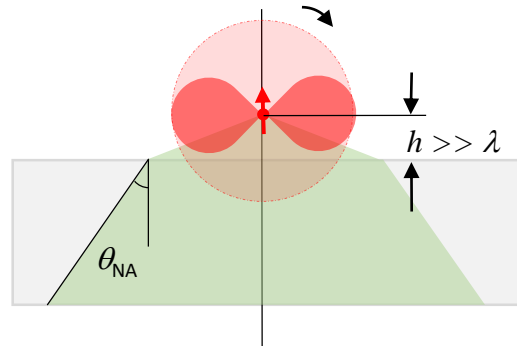
excitation

$$n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$$



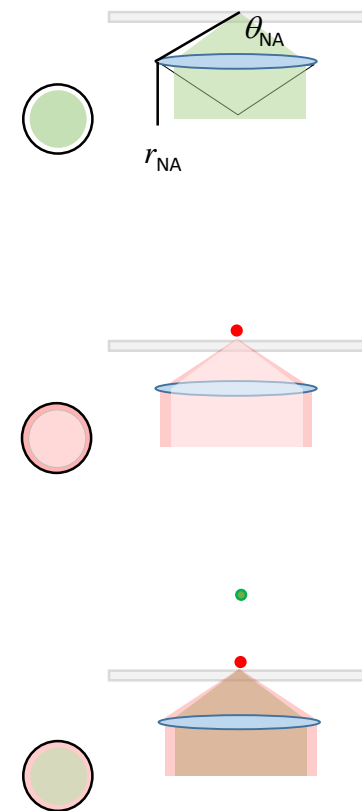
B

emission

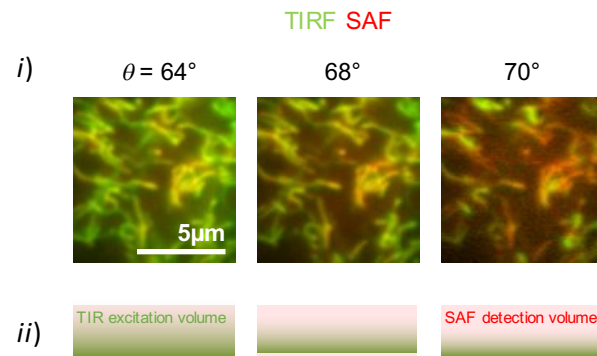


C

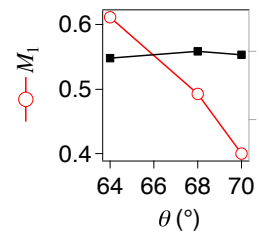
BFP image

fluorophore  $\rightarrow$  ●Oheim *et al.* Fig. 1

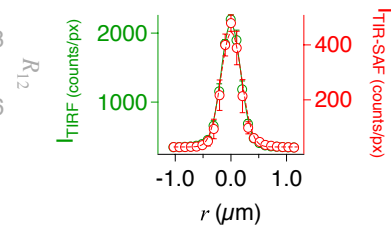
# A

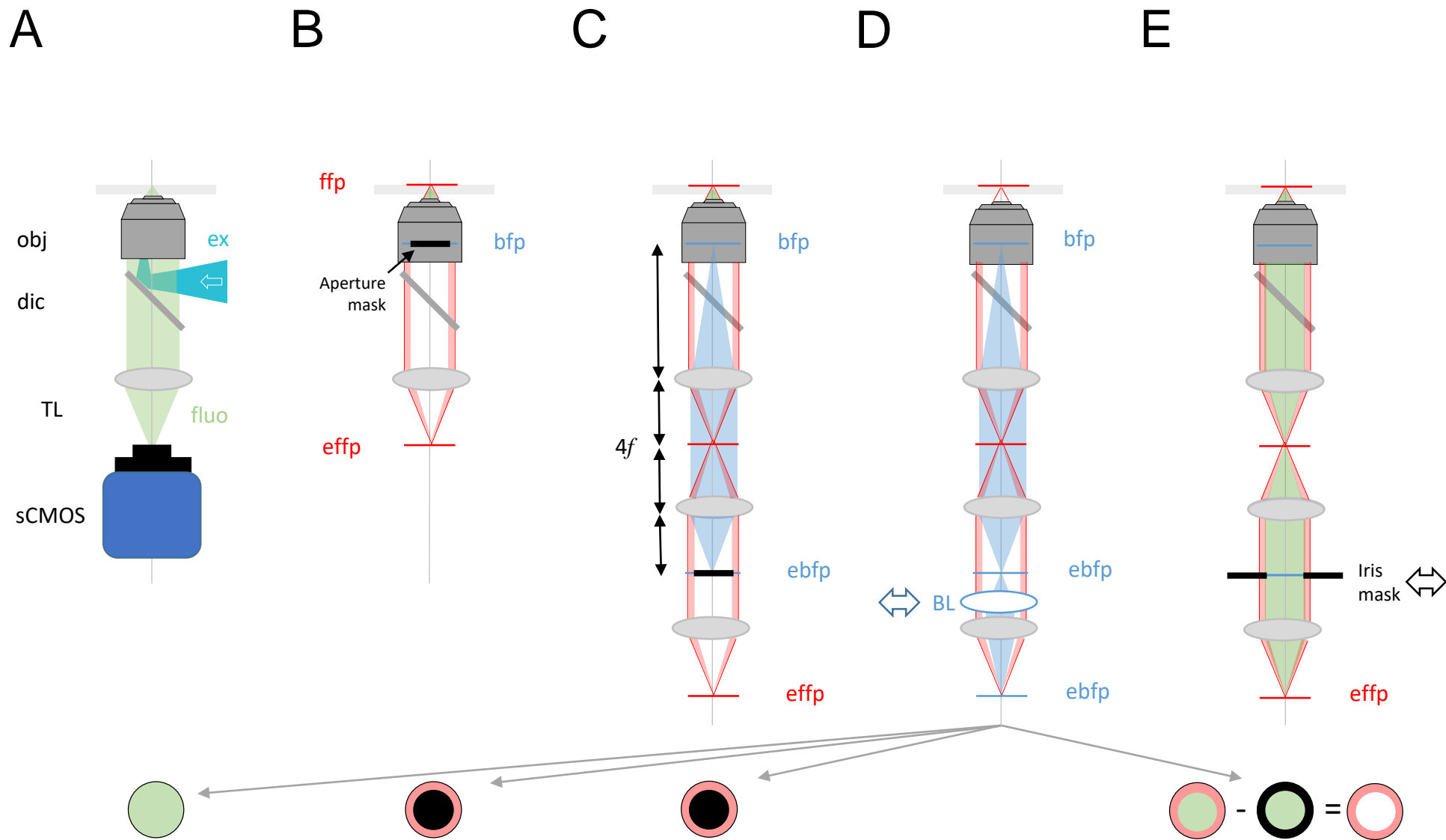


# B



# C





Oheim *et al.* Fig. 3