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Supercritical Angle Fluorescence Microscopy and Spectroscopy

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1 *Biophysical Review*

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3 **Supercritical angle fluorescence microscopy and spectroscopy**

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6 *Running title:* Fluorescence imaging from forbidden angles

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45 year 2018-19.

46

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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 FLUORESCENCE¹ (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 *“If you can see me, I can see you”*
105

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

¹ 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 δ is of the order of $\lambda/4$. Here λ
 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 2δ 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, Φ 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 θ_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° , 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 θ 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 θ (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 θ , **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 θ_{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 θ (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 θ , 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 optics², 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_{obj} is the objective's focal length, f_{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

² 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 ± 12 nm vs.
215 211 ± 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, $vSAF = (SAF + UAF) - UAF$, 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 θ_c because of the higher signal intensity, the
357 highest signal-to-bulk ratio was observed at much larger angles, at values above 68° 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 sucrose³ 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 NA_{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 θ are
380 sought for.

381

³ 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 ± 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 θ in objective-type TIRF microscopy. Similar to the earlier
411 RI-measurement, the approach is based on a series of θ_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

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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

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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° 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 ± 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 θ .
 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 ± 0.16 , C_M
730 $= 0.71$ and FWHM = 293 ± 2 nm for TIRF alone; vs. SBR = 14.4 ± 1.1 , $C_M = 0.88$, FWHM = 311 ± 1
731 nm for TIR-SAF. Thus, while no large difference was observed between FWHMs, the combined
732 optical sectioning of TIRF excitation and SAF detection almost tripled contrast ($\times 2.93$).

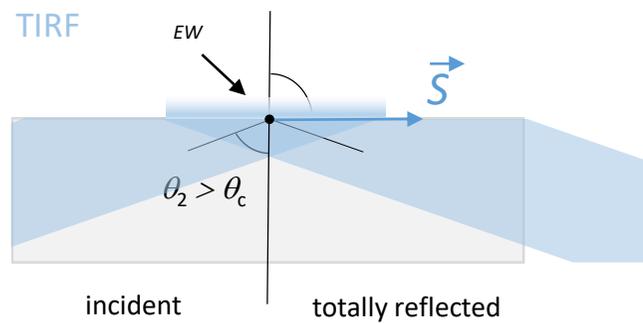
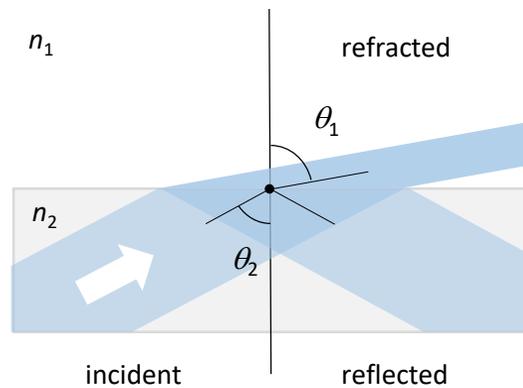
733

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

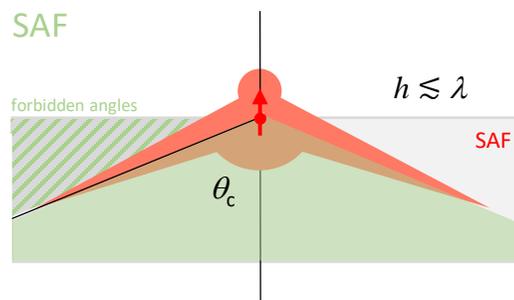
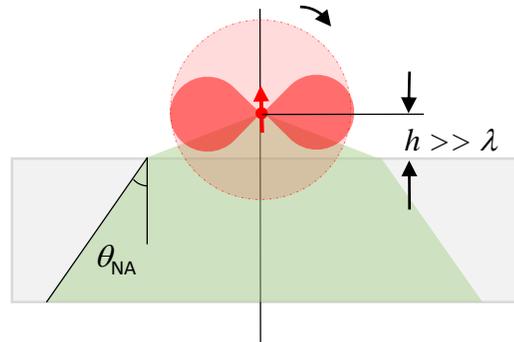
excitation

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



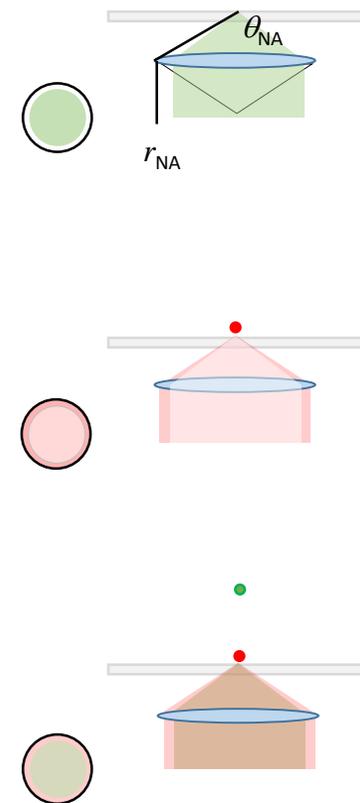
B

emission

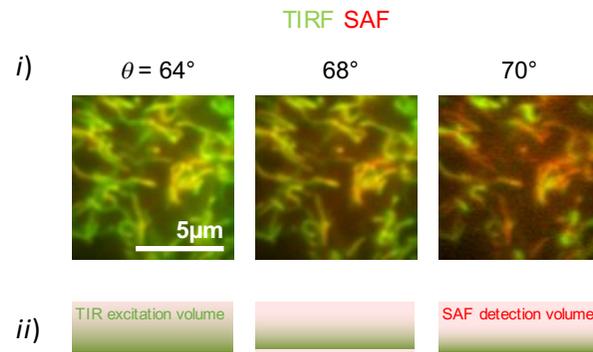


C

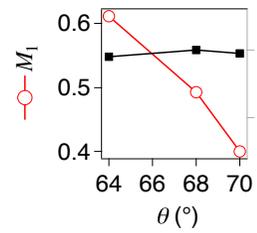
BFP image

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

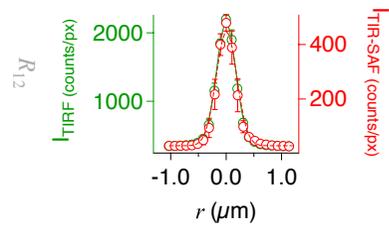
A

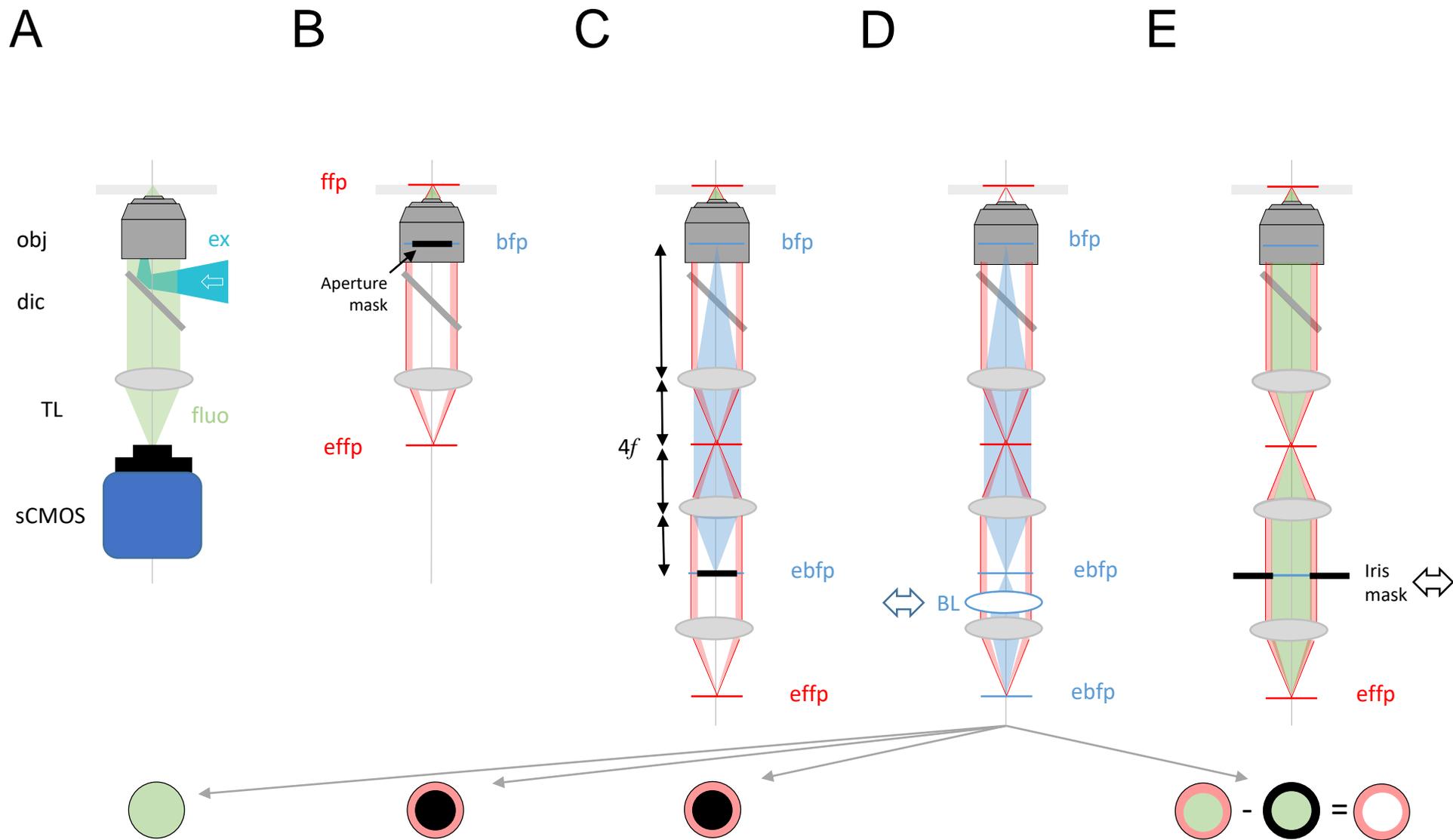


B



C





Oheim *et al.* Fig. 3