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# Indazole versus indole-based cationic merocyanines with red shifted in-cellulo emission for selective mitochondria imaging

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1 Indazole *versus* indole-based cationic  
2 merocyanines with red shifted *in-cellulo* emission  
3 for selective mitochondria imaging.  
4

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18 **Key words:** Fluorophore; Cellular biology; Indazole; Cationic merocyanine dyes; Mitochondria;

19

20 **Abstract**

21 Selective fluorophores are seminal for the development of fluorescent cell imaging allowing the  
22 simultaneous monitoring of several biological parameters. Mitochondria are one of the main targets  
23 of optical microscopy as their dynamics are related to many biological events. One of the best strategy  
24 to target them, is to design cationic dyes which accumulate preferentially in their membranes due to  
25 their high electrochemical potential. In this work, indazole scaffold was explored to build new cationic  
26 merocyanine dyes and compared with indole scaffold. This nitrogen heteroaromatic structure is still  
27 very uncommon in dyes chemistry; therefore, a lot has to be discovered about the effects of pH,  
28 solvent polarity and methylation in cyclic nitrogen on their optical properties. Finally, fluorescent  
29 imaging revealed that a red shift occurred in the emission of these molecules inside the mitochondria.

30

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## 32 **1 Introduction**

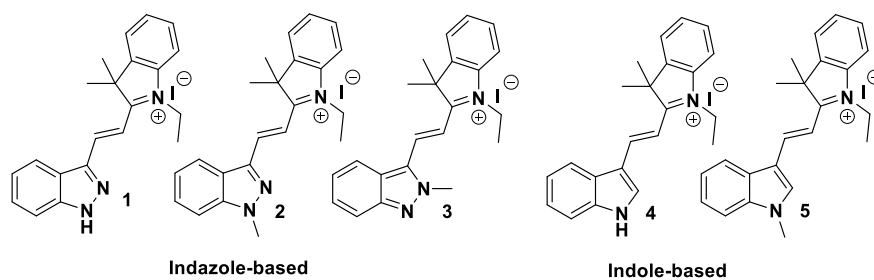
33 Mitochondria are one of the main targets of fluorescent bioimaging because their dynamic is related  
34 to numerous cellular processes and dysfunctions [1-3]; allowing evaluation of cells behavior. Cationic  
35 hydrophobic molecules are reported to accumulate preferentially in mitochondria, [4,5], therefore,  
36 cationic dyes are common in cell microscopy for their selective fluorescent imaging [6-24]. Indeed,  
37 their selectivity arise from the high membrane potential of this organelle, particularly in cancer cells  
38 [25,26], which retains lipophilic cations. This phenomenon was also exploited in photodynamic therapy  
39 to localize ROS generation inside tumor cells mitochondria [27-30]. This class of molecules also gave  
40 rise to the development of drugs known as “delocalized lipophilic cations” which showed cell toxicity  
41 by perturbing mitochondria membrane potential [31-33].

42 Merocyanine dyes are of common use in fluorescence imaging thanks to their high brightness in visible  
43 range and near infrared and their ability to sense their environment in response of several external  
44 stimuli such as polarity, H-bonding and viscosity [34-37]. These chromophores are constituted by an  
45 electron-donating group and an electron-withdrawing group linked by a polymethine chain featuring

46 an odd number of atoms. This arrangement provides them either a push-pull character making them  
47 good polarity probes or a cyanine character resulting in red shifted spectra. The balance between these  
48 two behaviors depends of the strength of their polarizing groups and of the polarization effect of their  
49 environment. Whereas indole is a classical electron-donating group in merocyanines [33,38], in this  
50 study, we chose to replace indole by indazole and to study the effect of this substitution. Indeed,  
51 indazole scaffold is drawing increasing attention as a key fragment for the conception of new drugs, as  
52 it is a bioisostere of indoles commonly found in bio-molecules. Replacing indole by indazole can be  
53 advantageous to tune the pharmacological profile and the affinity of a drug. Indeed, compared to  
54 indole, 1-*H*-indazole can interact more strongly with proteins thanks to its two successive nitrogen  
55 atoms able to create donor and acceptor hydrogen bonds [39-44]. Indazole scaffold has not yet been  
56 extensively exploited in chromophores design, consequently, little is known about its optical  
57 properties. On one hand, indazole is a less powerful electron-donating group than indole, but in the  
58 other hand, it should be more impacted by pH due to its more acid hydrogen. Moreover, nitrogen-  
59 containing aromatic compounds can feature very diverse optical properties as they can be used as  
60 electron-rich groups such as indoles or pyrroles, or as electron-withdrawing groups such as pyridine,  
61 pyridinium, quinoxalines. Nitrogen containing aromatic heterocycles can also favor intersystem  
62 crossing, generating triplet states through  $n-\pi^*$  orbitals population, leading to the development of  
63 photosensitizers [45]. On the other hand, nitrogen atom can act as hydrogen bond acceptor or donor  
64 modulating electron density along the unsaturated chain, making this class of compounds interesting  
65 to probe biological environment [46].

66 For those reasons, we turned our attention to the synthesis of indazole-based cationic merocyanine  
67 chromophores **1**, **2** and **3** that were designed with an indazole ring endowing the role of electron-  
68 donating group and an indolinium moiety as a charged electron-accepting group to promote  
69 mitochondria targeting (Fig. 1). Indole analogues **4** and **5** were also synthesized to provide a  
70 comparison.

71



72

73 Fig. 1. Structures of indazole and indole merocyanines.

74 Indazole merocyanines are expected to behave similarly to indole merocyanines, but with absorption  
75 and emission spectra slightly blue-shifted because indazoles are less electron rich. In the other hand,  
76 the presence of an acidic proton in the indazole aromatic ring ( $pK_a \approx 14$  [47] instead of  $pK_a \approx 17$  for indole  
77 in water) may generate interesting probing properties because this proton can be involved in hydrogen  
78 bonds with biomolecules (Fig. S1). Finally, it can be deprotonated making indazole a powerful electron-  
79 donating group in the same way as the phenol-related phenolate. In this work 1, or 2-methyl-1*H*-  
80 indazole derivatives 2 and 3, respectively, were synthesized as points of comparison to better  
81 understand the impact of tautomerism and deprotonation on the optical properties of these  
82 merocyanines.

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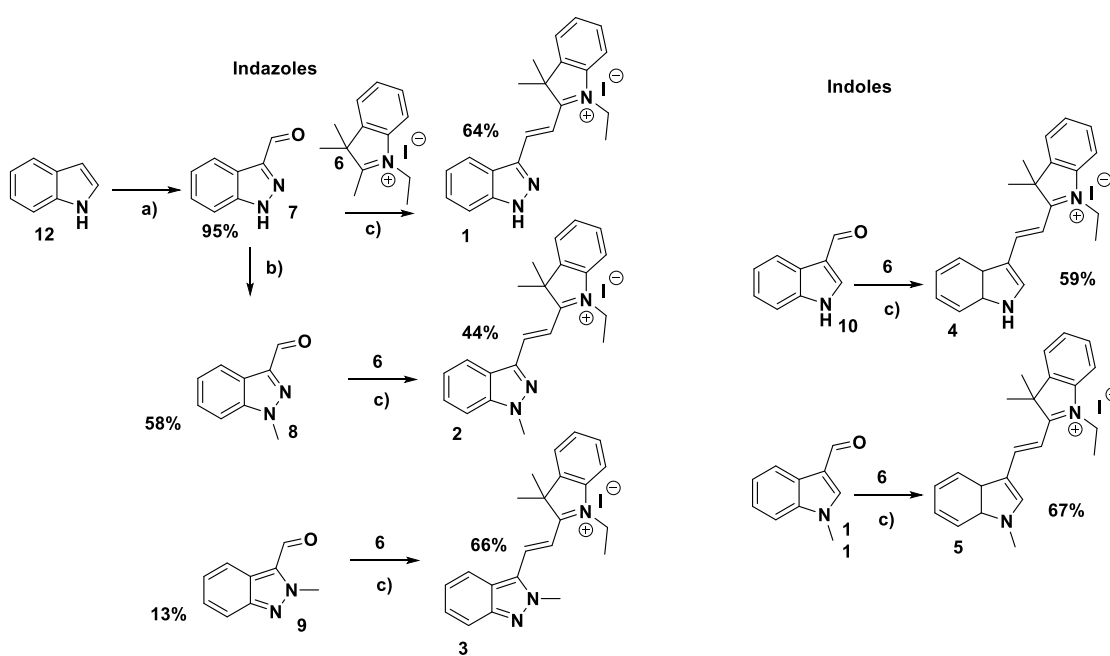
## 84 2 Results and discussion

### 85 2.1 synthesis

86 1 was readily prepared in two steps, starting from indole 12 (Scheme 1), the first reaction being a  
87 nitrosation providing in one step indazole-3-carboxaldehyde 7; this transformation was achieved in  
88 excellent yield according to our previously described protocol [48]. Then a Knoevenagel condensation  
89 was performed using 1-Ethyl-2,3,3-trimethyl-3*H*-indolium iodide 6 as the nucleophile to give the  
90 cationic dye 1 with 64% yield.

91 *N*-methylated products **8** and **9** were obtained jointly, as a separable mixture, by methylation of **7** with  
 92 methyl iodide and a weak base at 80 °C. This relatively high temperature promotes the formation of a  
 93 small amount of 2-methyl-1*H*-indazole-3-carbaldehyde **9** (13%) along with 1-methyl-1*H*-indazole-3-  
 94 carbaldehyde **8** as the major product (58%).[49] Then, these latter products were reacted with **6** in the  
 95 presence of piperidine to provide *N*-methylated dyes **2** and **3** in moderate 44% and 66% yields,  
 96 respectively. Indoles **4** and **5** were prepared according the same protocol starting from commercial  
 97 indoles **10** and **11**.

98



99

100 Scheme 1. Synthesis of cationic merocyanine dyes **1-5**. a) NaNO<sub>2</sub>, DMF, H<sub>2</sub>O, HCl, 0 °C then rt, 3h; b)  
 101 MeI, K<sub>2</sub>CO<sub>3</sub>, MeCN, 80 °C, 16h; c) piperidine (cat), MeCN, 80 °C 16h.

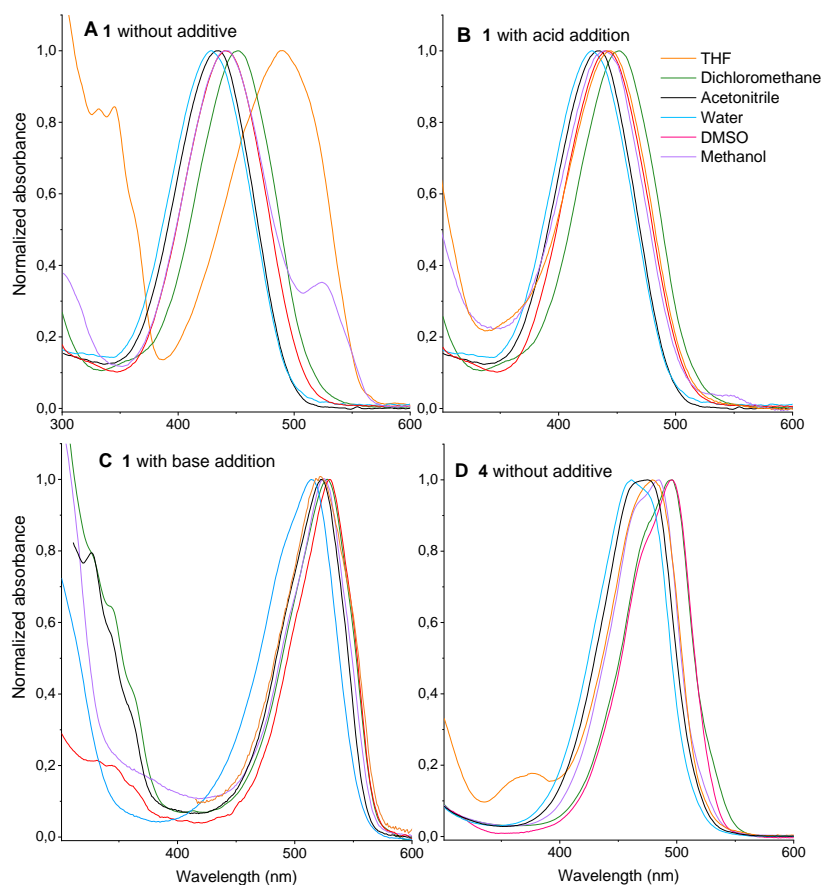
102

## 103 2.2 Optical properties

### 104 Absorption

105 Absorption of **1** was studied in various solvents (Fig. 2), the first results showed the presence of two  
106 distinct absorption bands, one with maximum around 450 nm and the other around 550 nm  
107 corresponding to two distinct species. Peculiarly, the results were non-reproducible, depending on  
108 solvents batches; the balance between these two bands being variable disclosing the fact that the pKa  
109 of **1** is likely to be very close to the neutrality. Therefore, spectroscopic studies were performed after  
110 addition of acetic acid, triethylamine or sodium hydrogenocarbonate in water (0.1% in weight) causing  
111 a total displacement of the equilibrium toward protonated or deprotonated form demonstrating that  
112 no tautomerism was involved in this phenomenon.

113 In acidic medium, **1** presents a negative absorption solvatochromism in accordance to the classical  
114 behavior of charged compounds; the spectral shape is large corresponding to the charge transfer of a  
115 push-pull chromophore. After addition of base, a 100 nm red shift is observed and the shape of the  
116 absorption band evolves to become more "cyanin-like", i.e. a red shifted sharper band. On the other  
117 hand, indole analogue **4** is less sensitive to pH variation. Indeed, in "pure solvent", only the protonated  
118 form is observed, and addition of acid (acetic acid) or base (trimethylamine) do not change the spectra  
119 (Fig. 2). The absorption maximum of indole derivative **4** is red shifted (460-490 nm) by comparison with  
120 protonated indazole **1** (430-460 nm), this demonstrates that, as anticipated, indole is a stronger  
121 electron-donating group. However, indazole compound **1** is more red shifted when deprotonated (515-  
122 520 nm). This observation suggests that indazole can be classified as less electro-donating when  
123 protonated than indole, but a stronger electro-donating group after deprotonation.



124

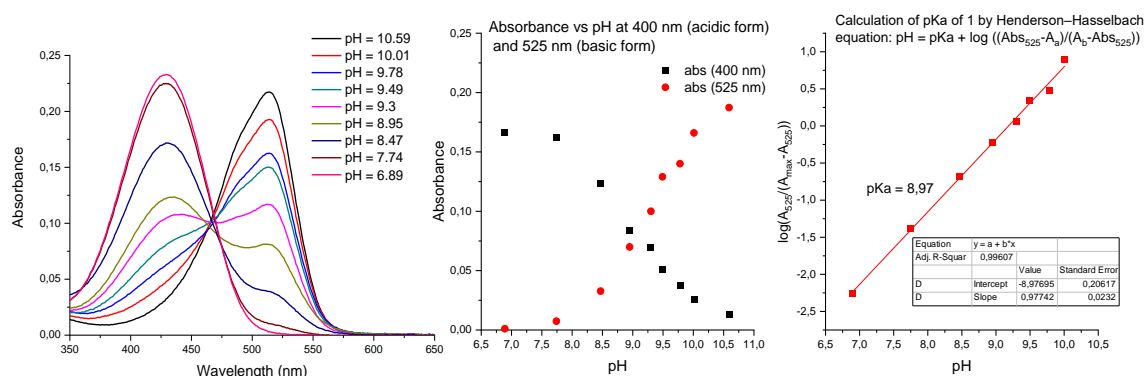
125 Fig. 2. Absorption solvatochromism of **1** at 21 °C in pure solvent (A) and effect of acid (B) or base  
 126 addition (C), and comparison with **4** in pure solvent (D). Water (blue), acetonitrile (black), DMSO  
 127 (red), methanol (mauve), dichloromethane (green), THF (orange).

128

129 The pKa of **1** was determined in PBS by carrying out absorption spectra in a pH range from 6.89 to  
 130 10.59 monitored by small addition of K<sub>2</sub>CO<sub>3</sub>. The presence of an isobestic point at 468 nm  
 131 demonstrated the progressive deprotonation of indazole **1** (Fig. 3). The absorption maximum in  
 132 function pH was plotted (Fig. 3) and Henderson-Hasselbach equation (equation S1) was used to  
 133 calculate the pKa, it was found to be close to 9.0 (Fig. 3). By comparison, the pKa of non-functionalized  
 134 indazole was reported to be 13.86 in water [47]. This pKa value is in accordance with the pH effect



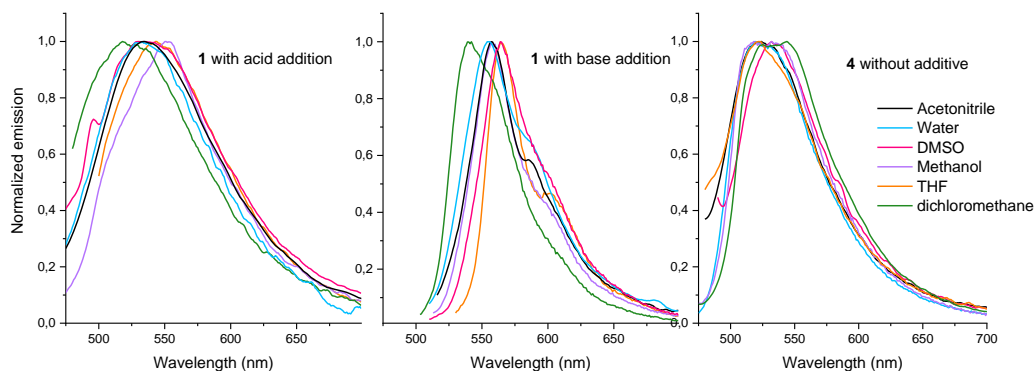
135 observed in organic solvents in which deprotonated form is more stabilized than the ionic protonated  
136 form.



137  
138 Fig. 3. Absorption of **1** in function of pH in PBS solutions and calculation of pKa by Henderson-  
139 Hasselbach equation at 525 nm: the ground state pKa of **1** can be read at y-intercept: 8.97 in PBS.

#### 140 Fluorescence

141 The profile of the fluorescence of merocyanine **1** was then studied in the same solvents (Fig. 4). In  
142 acidic medium, the fluorescence quantum yield is very low (0.02) in dichloromethane and no  
143 solvatochromism was observed (Fig. 4). In the basic form, fluorescence quantum yield of **1** was still  
144 very low (0.03) in dichloromethane, but the emission spectral shape was highly modified, it became  
145 more “cyanin like” i.e. with an emission band shifted to higher wavelengths with a shoulder in the red  
146 side and the solvatochromism was very weak. Unexpectedly, the fluorescence wavelength of indole **4**  
147 is very close to the one of protonated indazole **1** with maximum comprised between 525 nm and 550  
148 nm and the solvatochromism is negligible. As its absorption is red-shifted by comparison to **1**, the  
149 Stokes shift of **4** is very small ( $1633 \text{ cm}^{-1}$ ) indicating that the polarity of the fundamental and the  
150 emitting states are very close.



151

152 Fig. 4. Fluorescence solvatochromism of **1** at 21 °C with addition of acid (left), or base (right). Water  
 153 (blue), acetonitrile (black), DMSO (red), methanol (mauve), dichloromethane (green), THF (orange).

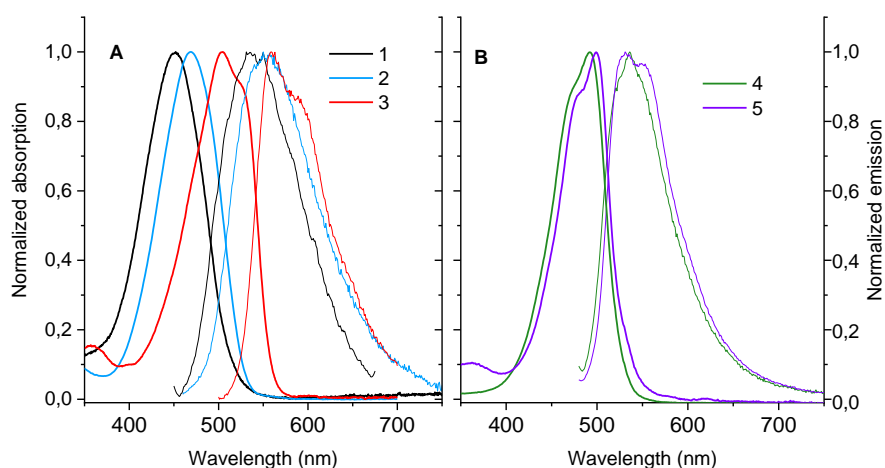
154

155 In the case of indazole **1**, in neutral medium (not shown), the fluorescence signals of both basic and  
 156 acidic forms were observed in similar ratio than absorption, meaning that no excited state  
 157 deprotonation occurred. The coexistence of the emissions of both forms suggests that emission is  
 158 faster than proton exchange, and therefore that the fluorescent lifetime of this molecule is likely to be  
 159 very short. Indeed, fluorescence decays were measured and proved to be short in dichloromethane  
 160 with a main component below 300 ps for both protonated and deprotonated forms (Fig. S2). Very short  
 161 fluorescence lifetimes were also measured for indole **4** below 300 ps. These results are in accordance  
 162 with previous measurements in structurally close compounds aztragon orange-R [50] and basic orange  
 163 21 [51] bearing indoles as donors. This low fluorescence might be related to the generation dark  
 164 “phantom” excited states in which fast deexcitation occurs due to the formation of twisted excited  
 165 states [52]. These results suggested that the fluorescence efficiency of these molecules can be  
 166 modulated by solvent viscosity.

#### 167 Comparison with methylated compounds

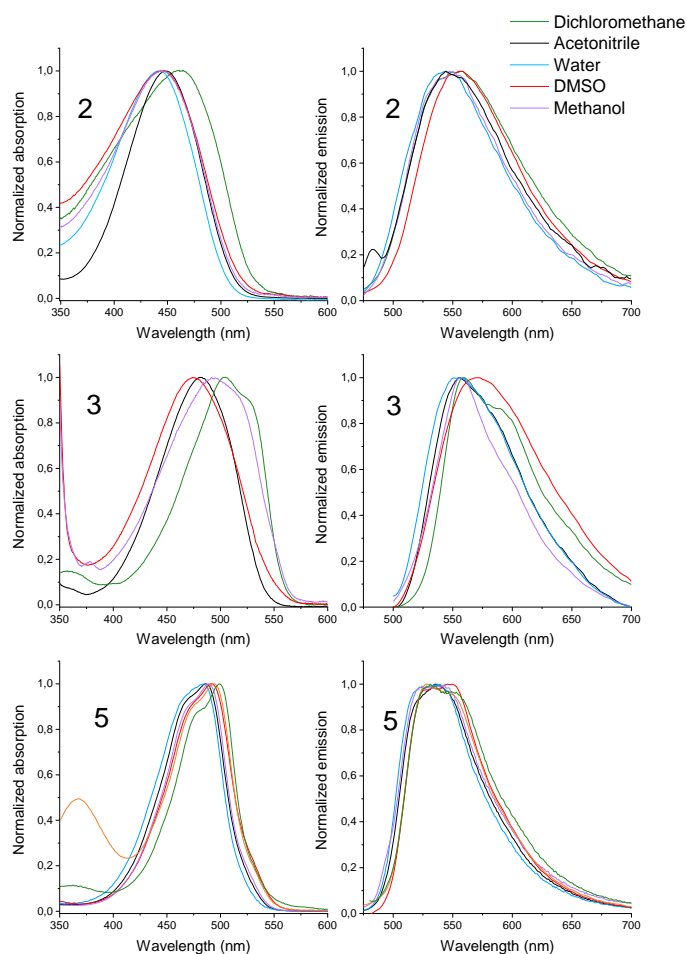
168 The optical properties of methylated compounds **2**, **3**, and **5** were then studied in dichloromethane  
 169 (Fig. 5). 1-methylated indazole **2** featured similar spectra shape to **1** but absorbed at 460 nm (vs 450

170 nm) and emitted at higher wavelength (551 nm vs 533 nm). This bathochromic shift was ascribed to  
171 the inductive effect of the methyl group making indazole a stronger donor, at a lesser extent, the same  
172 phenomenon was observed in absorption with indole **5** in comparison with indole **4** (5 nm red-shift).  
173 This demonstrates that the proton is localized on nitrogen in position 1 in indazole **1**. In comparison,  
174 2-methylated indazole **3** featured very different spectral shapes with absorption and emission red  
175 shifted, and a low Stokes shift ( $2020\text{ cm}^{-1}$  vs  $\approx 3500\text{ cm}^{-1}$ ); this behavior suggested that the molecule  
176 has more “cyanine” character. This phenomenon is related to the theoretical modelling done by  
177 Catalan, showing that 2-methylation of indazoles changes its electronic density making it more  
178 “quinoid” like, and less aromatic [47]. This loss of aromaticity in molecule **3** favors electron  
179 delocalization explaining its “cyanine” character [53]. The effect of the methylation of indole is very  
180 weak as the absorption and emission of **5** are very close to those of **4**. Indole compounds feature higher  
181 absorption wavelength than **1** and **2** and their Stokes shifts are very low ( $1600\text{ cm}^{-1}$  and  $1200\text{ cm}^{-1}$   
182 respectively for **4** and **5**), in addition, their absorption and emission band are symmetrical which is very  
183 typical of cyanine fluorophores. It is noticeable that the most red-shifted fluorophore in  
184 dichloromethane is indazole **3**, methylation of nitrogen in position 2 favors electrons delocalization by  
185 decreasing the aromaticity of indazole.



186

187 Fig. 5. Absorption and emission of (left) **1** (black), **2** (blue) and **3** (red) and (right) **4** (green) and **5**  
188 (violet) in dichloromethane at 21 °C.



190

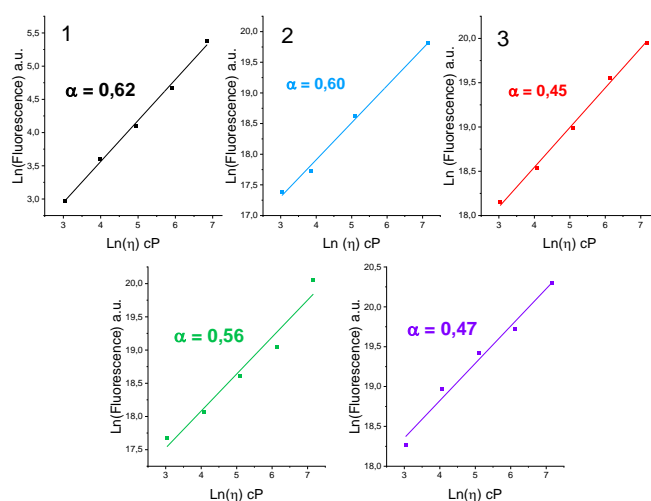
191 Fig. 6. Absorption and emission at 21°C of methylated compounds in water (blue), acetonitrile  
 192 (black), DMSO (red), methanol (mauve), dichloromethane (green), THF (orange).

193

### 194 2.3 Viscosity response

195 The viscosity dependence of the fluorescence was measured by varying the composition of an ethylene  
 196 glycol/glycerol mixture. This mixture was chosen to keep constant the solvent polarity during all the  
 197 experiment. The fluorescence  $\ln(F)$  as a function of viscosity  $\ln(\eta)$  was plotted (Fig. 7) and (Table 1), the  
 198 slope showed that dependence of the fluorescence of **1** upon viscosity (0,62) is high, slightly lower

199 than those measured with best commercial viscosity probes PicoGreen® (0,72) and SYBR Green I (0,75)  
 200 [54]. 1-methylated indazole **2** showed the same dependence (0.60), while 2-Methylated indazole **3**  
 201 featured a lower fluorescence response (0,45). By comparison, indoles featured slightly less  
 202 fluorescence dependence upon viscosity with slopes respectively at 0.56 for **4** and 0.47 for **5**. This  
 203 fluorescence dependence upon viscosity was high enough to make these compounds promising for  
 204 viscosity imaging in mitochondria [55].



205  
 206 Fig. 7. Fluorescence signal ( $\ln(F)$ ) of **1-5** in function of viscosity ( $\ln(\eta)$ ) in ethylene glycol/glycerol  
 207 mixtures.

208

	$\epsilon^a$	$\lambda_{\text{abs}}$ (nm)	$\lambda_{\text{em}}$ (nm)	Stokes shift <sup>b</sup>	$\phi_F^c$	viscosity slope
<b>1</b>	34 000	450	533	3460	0.02	0.62
<b>2</b>	23000	460	551	3590	0.01	0.60
<b>3</b>	30 000	503	560	2020	0.02	0.44
<b>4</b>	46000	492	535	1633	0.01	0.56
<b>5</b>	44000	499	532	1243	0.01	0.47

210 Table 1. Spectroscopic data of **1-5** in dichloromethane at 21 °C. [a] molar extinction given in (mol<sup>-1</sup>.L.cm<sup>-1</sup>), [b] Stokes shift given in cm<sup>-1</sup>, [c] fluorescence quantum  $\phi_F$  yield was measured using  
211  
212 coumarine 153 ( $\phi_F$  =0.54) in ethanol as reference [56].

213

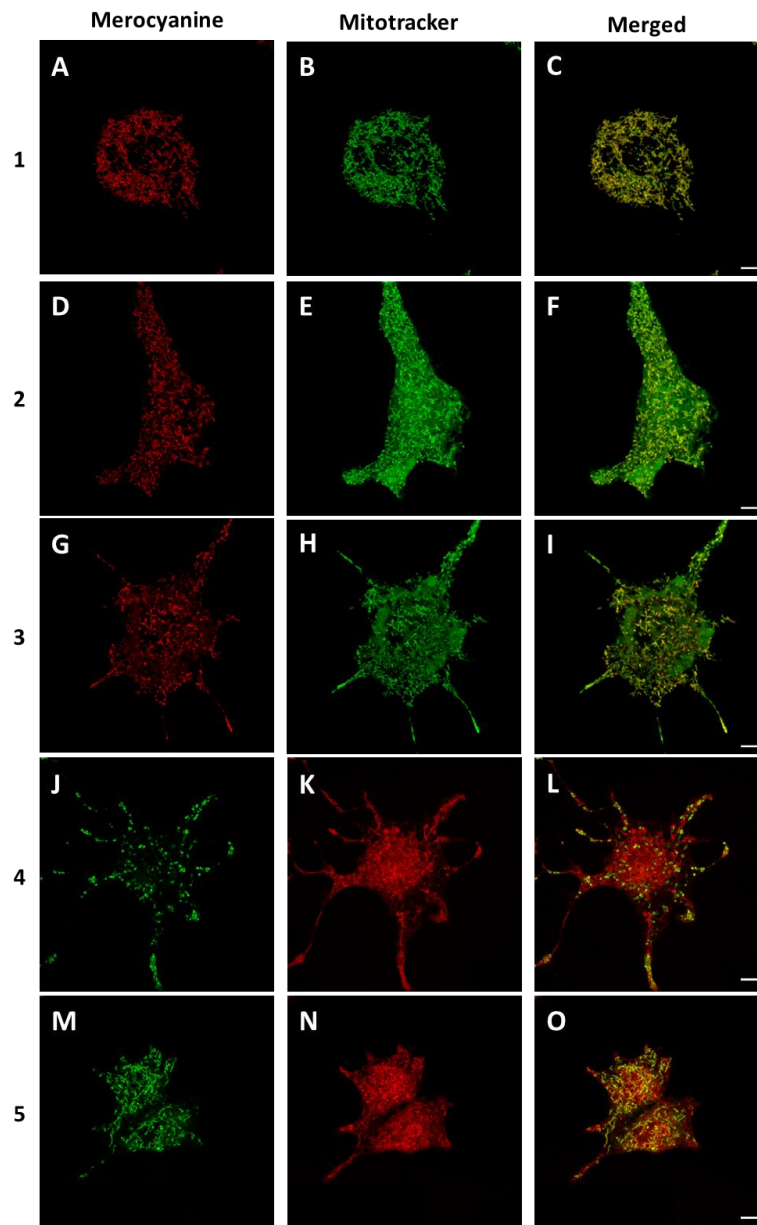
### 214 **3 Fluorescence imaging**

215

#### 216 **3.1 Fluorescence images**

217 Fluorescence images were recorded in tumoral PC12 cells. At high magnification (63x objective, NA  
218 1.4), xyz acquisitions revealed that incubation with **1**, **2** or **3** (10<sup>-6</sup> M, 45 min) induced a combination of  
219 numerous moderately stained filamentous structures and sparse hot spots in the entire cytoplasm (Fig.  
220 8 A, D, G). Intensively fluorescent spots were either linked to filamentous structures or isolated. In  
221 contrast, plasma and nuclear membranes were devoid of labeling (Fig. 8 A, D, G).

222 In addition, double-labeling experiments indicated that indazole based-merocyanines **1**, **2** or **3** co-  
223 localized strongly with mitochondria labeling (MitoTracker™ Green) both for filamentous and spot-  
224 shape structures (Fig. 8) (Pearson correlation coefficients: 0.827, 0.838 and 0.756 respectively for **1**, **2**  
225 and **3**). The fluorescence localization in cells of indole derivatives **4** and **5** was also studied; co-  
226 localization was implemented with MitoTracker™ Red since indole compounds emission overlapped  
227 with MitoTracker™ green emission. Unexpectedly, these two molecules did not localize in the same  
228 cell compartment, **4** was not able to reach mitochondria and featured a liposomal localization, while **5**  
229 localized selectively in mitochondria similarly to indazole compounds.



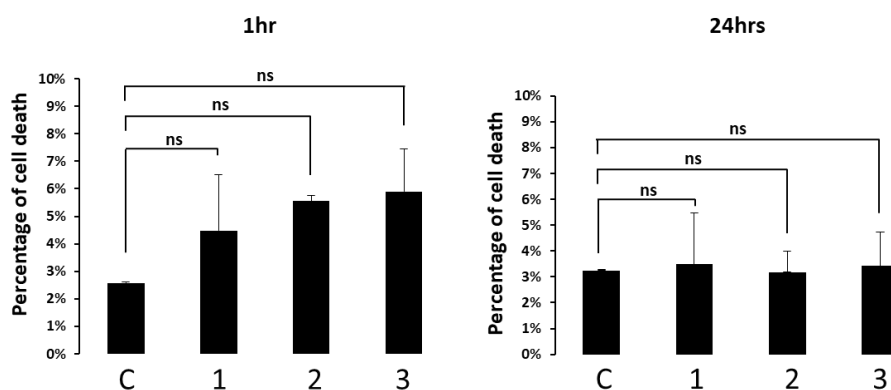
230

231 Fig. 8. Subcellular staining of living PC12 cells with **1** - **5** through confocal microscopy. Single staining  
 232 (Left). Single staining for the mitochondria marker MitoTracker™ Green (panels B, E, H) and  
 233 MitoTracker™ Red (Panels K, N). Double staining with **1**, **2** or **3** and MitoTracker™ Green (panels C, F,  
 234 I) and **4** or **5** with MitoTracker™ Red (Panels L, O). Scale bar 10 μm

235

236 **3.2 cytotoxicity**

237 A fluorescent membrane integrity test with the NucGreen™ reagent indicated that 1- or 24-hrs  
238 incubation with **1**, **2** or **3** ( $10^{-6}$ M) did not alter viability of PC12 cells suggesting that merocyanine  
239 compounds were not cytotoxic at this concentration (Fig. 9). Indoles cytotoxicity was also studied and  
240 similar results were obtained (SI Fig. S5).



241  
242 Fig. 9. Effect of merocyanine compounds ( $10^{-6}$  M) on cell viability. Measurement of the cytotoxic effect  
243 on PC12 cells after exposure with **1**, **2** or **3** during 1 hr or 24 hrs through fluorescent membrane integrity  
244 test. Each value represents the mean of the percentage of dead cell ( $\pm$  sem) on at least 150 cells. n.s.,  
245 no statistical significance versus control.

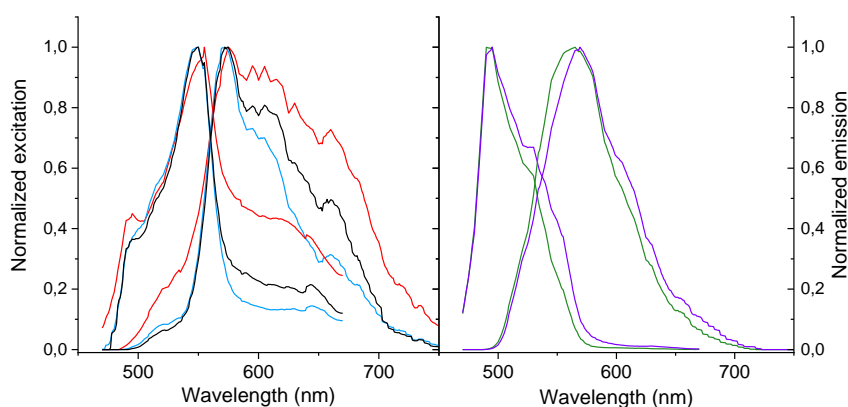
### 246 247 **3.3 In cellulo spectra**

248 *In cellulo* excitation and emission spectra were recorded for each compound (Fig. 10). Surprisingly, for  
249 indazoles compounds, they were very distinct from spectra in cuvettes; they both featured very similar  
250 spectra with an excitation maximum at 540 nm and fluorescence maximum at 580 nm corresponding  
251 to a 20-40 nm red shift by comparison with fluorescence emission in pure solvent. In the case of **1** the  
252 red shift might be the result of a deprotonation inside of mitochondria, however, excitation and  
253 emission are a little bit more shifted than previously observed in basic medium (see Fig. 4). Moreover,  
254 non-deprotonable **2** and **3** featured the same excitation and emission wavelengths as **1** indicating that  
255 this fluorescence wavelength shift does not originate from deprotonation.



256 *In cellulo* emissions of indole compounds **4** and **5** were also red shifted with a maximum at 550-560  
257 nm in comparison with water and organic solvent (530-540 nm) (Fig. 10), but their excitation spectra  
258 matched their absorbance measured in cuvette. These red shifts are clearly smaller than those of  
259 indazole compounds, resulting that, *in cellulo*, indazole compounds are more red-shifted than indoles.  
260 This behavior can be only explained by an uncommon effect affecting the optical properties of  
261 indazoles, which does not occur with indoles.

262



263

264 Fig. 10. *In cellulo* and excitation and emission spectra of indazoles (left) **1** (black), **2** (blue), **3** (red)  
265 and indoles (right): **4** (green), **5** (violet).

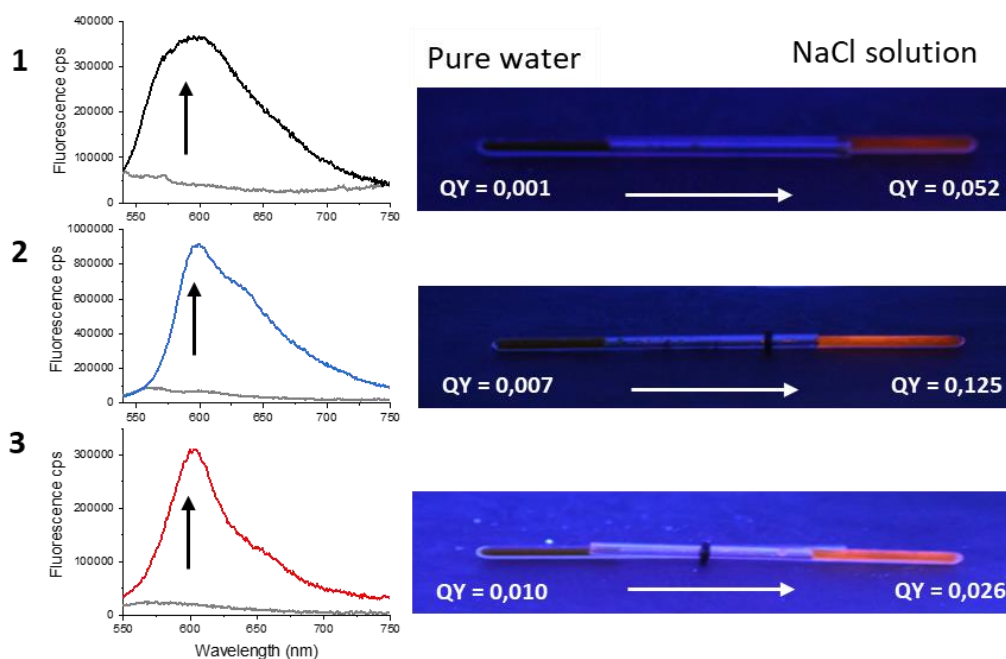
266

267 Merocyanine color change can be induced by chemical reactions, especially, indolinium group is prone  
268 to photooxidation [57] or can suffer from nucleophilic addition of hydroxide ion [58,59] or of cyanide  
269 ion even in mild conditions [60-66]. However these reactions lead to blue shifts in absorption and  
270 emission due to conjugation breaking. Red shift was described in the case of aggregate formation,  
271 particularly J-aggregates; this phenomenon was first observed in mitochondria tagged with JC-1  
272 cyanine dye [67]. Indeed, in the presence of a surfactant or with increasing ionic strength, many  
273 cyanines form monolayer of J-aggregates [68,69]. This phenomenon gives rise to a red shifted very  
274 sharp absorption and emission bands featuring a very weak Stokes shift resulting from cooperative

275 intermolecular connection of electronic density. It had been used to probe mitochondria membrane  
276 potential since more polarized mitochondrial membranes accumulate more lipophilic cationic  
277 dyes.[70]

278 To challenge this hypothesis, the formation of aggregates was probed by adding an aqueous saturated  
279 solution of NaCl to a water solution of **1**, **2** or **3** (final dye concentration  $5 \text{ mg}\cdot\text{ml}^{-1}$  in 3M NaCl) inside  
280 of quartz capillaries. Under UV lamp exposition, no visible fluorescence appeared in pure water while  
281 the NaCl solution became orange (Fig. 11). The fluorescence band (excitation 520 nm) was recorded  
282 with an integrating sphere in both media and a stunning exaltation was observed with all compounds  
283 (Fig. 11). The fluorescence quantum yields (QY) of molecules **1**, **2** and **3** measured with an integrating  
284 sphere reaffirmed this exaltation (Fig. 11), with molecule **1** going from fluorescence quantum yield QY  
285 = 0.001 in pure water to QY= 0.052 in NaCl solution, molecule **2** from QY= 0.007 to QY = 0.125. On the  
286 other hand, the fluorescence quantum yield change was less pronounced with molecule **3** (from  
287 QY=0.01 to QY=0.025) (Fig. 11). It is worth noting that these molecules were only partly aggregated,  
288 and a large amount remained soluble, leading to light absorption by free molecules and thus to  
289 fluorescence quantum yield underestimation; this effect was more prominent with molecule **3** because  
290 the absorption of free molecule is the most red-shifted. In order to avoid this excitation filter effect,  
291 using more diluted solutions was tested (final dye concentration  $0,3 \text{ mg}\cdot\text{ml}^{-1}$ ) but no or not enough  
292 aggregation occurred in order to detect aggregates emission. In our experiments, the full width at half  
293 maximum (FWHM) of the emissions remained quite large in comparison of what is commonly observed  
294 when J-aggregates are formed in surfactant monolayers. This is probably because the arrangement of  
295 the aggregates was not well ordered enough, and that a mixture of several aggregation patterns was  
296 obtained leading to larger emission bands. Moreover, the measurement of the absorption of these  
297 aggregates was not successful as there was not a distinct red-shifted absorption band, probably  
298 because only a very small part of aggregated compounds features a J-aggregate structure. As these  
299 merocyanines did not promote structurally-pure enough aggregates, introducing different side chains  
300 on these compounds may lead to a more selective molecular packing. [71]

301

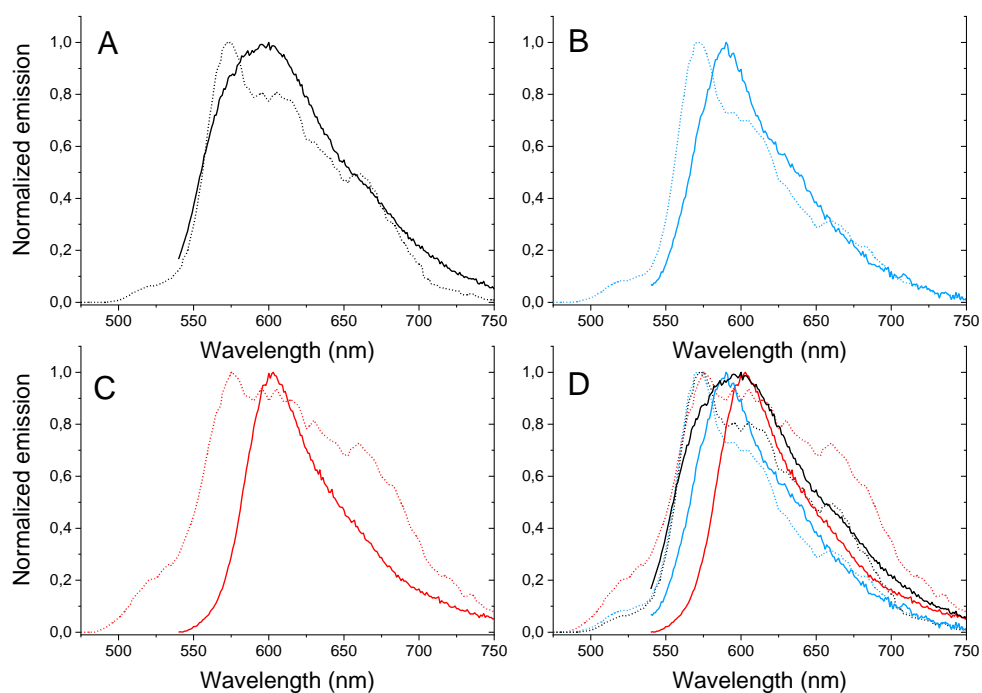


302

303 Fig. 11. Emission of **1**, **2** and **3** in pure water (grey) and in NaCl (3M) and corresponding photos of  
304 capillaries exposed to a 360 nm UV-lamp. Fluorescence quantum yield (QY) were estimated in an  
305 integrating sphere at 21 °C.

306

307 Aggregates spectra were then superimposed with fluorescence signal measured *in cellulo* (Fig. 12). The  
308 wavelength of the aggregates emission proved to be close to the emission observed "*in cellulo*" which  
309 supports the hypothesis of aggregates formation inside the mitochondria. In addition, emission spectra  
310 were also recorded for the solid powders of **1**, **2** and **3** and no detectable fluorescence was measured  
311 (Fig. S3) demonstrating that fluorescence arises from a the particular dye aggregation observed in NaCl  
312 solutions. Interestingly, methylated products **2** and **3** featured sharper emission than non-methylated  
313 **1**, as if aggregates formed with **1** were a mixture of both 1H and 2H tautomers.



314

315 Fig. 12. Emission of aggregates (solid line) of **1** (A), **2** (B) and **3** (C) in a solution of NaCl (3M) and  
 316 superimposition (D) with *in cellulo* emission (dotted line).

317 The same experiments were carried out with indole compounds, fluorescence was detected upon  
 318 aggregation, but the intensity of this signal was very weak and no fluorescence quantum yield could  
 319 be measured. The emission of aggregates and *in cellulo* emission spectra did not superimpose,  
 320 suggesting that the emission of indole compounds **4** and **5** in cells is not related to an aggregation  
 321 effect (Fig. S4).

### 322 **3 Conclusion**

323 In this work, indazole was proposed for the first time as an electron-donating group to build cationic  
 324 merocyanine dyes. These molecules were easily synthesized and featured excellent ability to probe  
 325 viscosity in polar environment by comparison with commercial dyes. Their optical properties were  
 326 compared with those of indole merocyanines, and it appeared that indazoles are more pH sensitive,

327 and that a small modification such as methylation of nitrogen in position 2 can fundamentally change  
328 their optical properties allowing switching from push-pull to cyanine fluorophores.

329 Inside cells, indazoles compounds **1-3** provided selective fluorescence images of mitochondria as  
330 evidenced by co-labeling experiments. While an increased fluorescence was expected in mitochondria  
331 due to the viscosity of their membrane, indazole merocyanines also featured surprising red shifted  
332 excitation and emission spectra. This red shift is not correlated to viscosity, but probably results from  
333 the formation of J-aggregates inside of mitochondria as previously reported with other cationic dyes.  
334 To support the formation of this particular supra molecular arrangement, it was checked that these  
335 molecules feature no solid-state emission. However, it was not possible to fully demonstrate the  
336 formation of J-aggregates as the full widths at half maximum of emission spectra were too large, and  
337 we were unable to measure absorption spectra of these aggregates. This apparent red shift in  
338 mitochondria is very valuable for fluorescent imaging because it permits to trustworthy discriminate  
339 dyes inside the mitochondria from cytoplasmic dyes, and it shifts dyes absorption and emission closer  
340 to the biological transparency window.

341

## 342 **4 Experimental section**

343

### 344 **4.1 Synthesis**

345 Material and methods: Indole 99% was purchased from Acros Organics, and solvents from Fisher  
346 scientific. Column chromatography purifications were performed on silica gel (40–63  $\mu\text{m}$ ). Thin-layer  
347 chromatography (TLC) analyses were carried out on Merck DC Kieselgel 60 F-254 aluminum sheets.  
348 The spots were visualized through illumination with UV lamp ( $\lambda = 254 \text{ nm}$  and  $360 \text{ nm}$ ). IR spectra were  
349 recorded with a universal ATR sampling accessory.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra ( $^{13}\text{C}$ APT or  $^{13}\text{C}$ CPD  
350 experiments) were recorded on a 300 MHz spectrometer. Chemical shifts are expressed in parts per

351 million (ppm) from the residual non-deuterated solvent signal contained in CDCl<sub>3</sub> ( $\delta$ H = 7.26,  $\delta$ C =  
352 77.16), in acetone-d<sub>6</sub> ( $\delta$ H = 2.05,  $\delta$ C = 29.84) and in DMSO-d<sub>6</sub> ( $\delta$ H = 2.50,  $\delta$ C = 39.52). Multiplicities  
353 are described as s (singlet), d (doublet), t (triplet), brs (broad peak) etc. Coupling constants, J values,  
354 are reported in Hz. High-resolution mass spectra (HRMS) were obtained using an orthogonal  
355 acceleration time-of-flight (oa-TOF) mass spectrometer equipped with an electrospray source and in  
356 the positive and negative modes (ESI+/-).

357

358 **1H-indazole-3-carbaldehyde:** The product was obtained following the protocol previously described  
359 by our team.[15]

360 To a solution of NaNO<sub>2</sub> (550 mg, 8 mmol, 8 eq.) in 4 mL of deionized water and 3 mL of DMF at 0 °C  
361 was added slowly HCl (1.33 mL of 2 N aq., 2.7 mmol, 2.7 eq.) and the resulting mixture was kept under  
362 argon for 10 min. A solution of indole (117 mg, 1 mmol, 1 eq.) in DMF (1 mL) was then added at 0 °C  
363 over a period of 2 h using a syringe pump. After addition, the reaction was stirred 3 additional hours  
364 at room temperature or until disappearance of intermediate products. The product was purified by  
365 column chromatography on silica gel, eluting with petroleum ether/EtOAc, 8 : 2 to provide the pure  
366 compound as a white solid (145 mg, 99%). Silica gel TLC R<sub>f</sub> 0.37 (petroleum ether/EtOAc, 3 : 2); **<sup>1</sup>H NMR**  
367 **(SO(CD<sub>3</sub>)<sub>2</sub>, 300 MHz):**  $\delta$  (ppm) 14.17 (brs, 1H), 10.20 (s, 1H), 8.14 (d, *J* = 8.5 Hz, 1H), 7.70 (d, *J* = 8.5 Hz,  
368 1H), 7.49 (dt, *J* = 7.0, 1.0 Hz, 1H), 7.37 (dt, *J* = 7.0, 1.0 Hz, 1H); **<sup>13</sup>C NMR (SO(CD<sub>3</sub>)<sub>2</sub>, 75 MHz)**  $\delta$  : 187.4,  
369 143.4, 141.1, 127.3, 123.8, 120.7, 120.2, 111.2; **IR:**  $\nu$  (cm<sup>-1</sup>) = 3254, 3174, 1671, 1458, 1331, 1251, 1092,  
370 792, 739; **HRMS (ESI<sup>-</sup>):** found = 145.0402, C<sub>8</sub>H<sub>5</sub>N<sub>2</sub>O [M-H]<sup>-</sup> = 145.0390. **m.p.:** 141 °C.

371

372 **1-methyl-1H-indazole-3-carbaldehyde 6:** 1H-indazole-3-carbaldehyde (730 mg, 5 mmol) in dry MeCN  
373 (5 mL) was treated with iodomethane (0.50 mL, 1.5 eq.) in presence of K<sub>2</sub>CO<sub>3</sub> (1.0 g, 1.5 eq.). The  
374 solution was refluxed for 16 h, then neutralized with a solution NH<sub>4</sub>Cl and extracted 3 times with EtOAc.  
375 The crude mixture was composed of N<sub>1</sub> and N<sub>2</sub> methylated products with a ratio of 75:15 respectively

376 (determined by  $^1\text{H}$  NMR spectroscopy). Both isomers were isolated by flash chromatography  
377 (cyclohexane: DCM: EtOAc 65:30:5) (464 mg, white amorphous solid, 58% isolated yield).  $^1\text{H}$  NMR  
378 ( $\text{CDCl}_3$ , 300 MHz):  $\delta$  (ppm) = 10.23 (s, 1H), 8.31 (dt, 1H,  $J$  = 8.1, 1.0 Hz), 7.54 – 7.45 (m, 2H), 7.37 (m,  
379 1H,  $J$  = 7.9, 5.5, 2.3 Hz), 4.20 (s, 3H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75 MHz):  $\delta$  (ppm) = 186.7, 143.1, 141.5, 127.6,  
380 124.2, 122.4, 122.2, 109.5, 36.7. IR:  $\nu$  ( $\text{cm}^{-1}$ ) = 3059, 2949, 2792, 1671, 1474, 1426, 1254, 1060, 788,  
381 749, 741. HRMS (ESI $^+$ ): found = 161.07159; calculated  $[\text{M}+\text{H}]^+ = [\text{C}_9\text{H}_9\text{N}_2\text{O}]^+ = 161.07149$ . m.p.: 62-63  
382  $^\circ\text{C}$ .

383  
384 **2-methyl-2H-indazole-3-carbaldehyde 7**: See 1-methyl-1H-indazole-3-carbaldehyde (104 mg, white  
385 solid, 13% isolated yield).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz):  $\delta$  (ppm) = 10.28 (s, 1H), 8.03 – 7.94 (m, 1H), 7.85  
386 – 7.77 (m, 1H), 7.42 – 7.30 (m, 2H), 4.48 (s, 3H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75 MHz):  $\delta$  (ppm) = 177.5, 147.6,  
387 130.5, 126.8, 126.4, 125.2, 118.7, 118.3, 40.9. IR:  $\nu$  ( $\text{cm}^{-1}$ ) = 2954, 2833, 1673, 1457, 1431, 1291, 1191,  
388 1048, 780, 743. HRMS (ESI $^+$ ): found = 161.07128; calculated  $[\text{M}+\text{H}]^+ = [\text{C}_9\text{H}_9\text{N}_2\text{O}]^+ = 161.07149$ . m.p.:  
389 65-66  $^\circ\text{C}$ .

390  
391 **General procedure: (Knoevenagel condensation)** Both the aldehyde (1 eq.) and 1-ethyl-3,3-  
392 dimethyl-3H-indolium iodide **4** (1 eq.) were solubilized in dry MeCN (final concentration of 1 M).  
393 Piperidine (10 mol%) was added and the solution was heated to 80  $^\circ\text{C}$  for 6 hours to 16 hours. The  
394 solvent was removed under reduced pressure and the crude mixture was purified by recrystallization  
395 in diethyl ether.

396  
397  
398 **2-(2-(1H-indazol-3-yl)vinyl)-1-ethyl-3,3-dimethyl-3H-indolium iodide 1**: The product was synthesized  
399 following the *general procedure* with 1H-indazole-3-carbaldehyde **5** (100 mg, 0.68 mmol) and 1-ethyl-  
400 2,3,3-trimethyl-3H-indolium iodide **4** (215 mg, 1 eq) (193 mg, pink solid, 64% isolated yield).  $^1\text{H}$  NMR  
401 ( $\text{SO}(\text{CD}_3)_2$ , 300 MHz):  $\delta$  (ppm) = 14.43 (s, 1H), 8.67 (d,  $J$  = 16.3 Hz, 1H), 8.46 (d,  $J$  = 8.1 Hz, 1H), 8.03 –

402 7.89 (m, 2H), 7.80 – 7.60 (m, 4H), 7.56 (t,  $J = 7.7$  Hz, 1H), 7.45 (t,  $J = 7.4$  Hz, 1H), 4.73 (q,  $J = 7.0$  Hz, 2H),  
403 1.86 (s, 6H), 1.52 (t,  $J = 7.2$  Hz, 3H).  $^{13}\text{C NMR (SO(CD}_3)_2, 75 \text{ MHz)}$ :  $\delta$  (ppm) = 181.0, 144.0, 143.8, 141.7,  
404 140.6, 140.5, 129.4, 129.2, 127.4, 123.5, 123.1, 122.4, 121.0, 115.1, 111.6, 111.3, 52.3, 42.2, 25.7, 13.5.  
405 **IR**:  $\nu$  ( $\text{cm}^{-1}$ ) = 3077, 3028, 2973, 1599, 1577, 1533, 1433, 1235, 1098, 958, 772, 748. **HRMS (ESI<sup>+</sup>)**: found  
406 = 316.1827; calculated  $[\text{M}]^+ = 316.1814$ . **m.p.**: 193-195 °C.

407

408 **1-ethyl-3,3-dimethyl-2-(2-(1-methyl-1H-indazol-3-yl)vinyl)-3H-indolium iodide 2**: The product was  
409 synthesized following the *general procedure* with 1-methyl-1H-indazole-3-carbaldehyde **6** (15 mg, 0.1  
410 mmol) and 1-ethyl-2,3,3-trimethyl-3H-indolium iodide **4** (30 mg, 1 eq) (20 mg, dark pink solid, 44%  
411 isolated yield).  $^1\text{H NMR (CDCl}_3, 300 \text{ MHz)}$ :  $\delta$  (ppm) = 8.45 (d,  $J = 16.2$  Hz, 1H), 8.42 – 8.36 (m, 1H), 7.84  
412 – 7.78 (m, 1H), 7.74 (d,  $J = 16.2$  Hz, 1H), 7.65 – 7.53 (m, 6H), 5.01 (q,  $J = 7.4$  Hz, 2H), 4.25 (s, 3H), 1.91  
413 (s, 6H), 1.69 (t,  $J = 7.4$  Hz, 3H).  $^{13}\text{C NMR (CDCl}_3, 75 \text{ MHz)}$ :  $\delta$  (ppm) = 180.8, 144.8, 143.3, 142.0, 140.6,  
414 139.9, 130.04, 130.00, 128.1, 125.4, 123.8, 122.83, 121.73, 115.4, 111.2, 110.5, 52.6, 44.9, 37.2, 27.2,  
415 14.1. **IR**:  $\nu$  ( $\text{cm}^{-1}$ ) = 3003, 2976, 1592, 1534, 1455, 1311, 1237, 1065, 750. **HRMS (ESI<sup>+</sup>)**: found =  
416 330.1975; calculated  $[\text{M}]^+ = 330.1970$ . **m.p.**: decomposition > 195 °C.

417

418 **1-ethyl-3,3-dimethyl-2-(2-(2-methyl-2H-indazol-3-yl)vinyl)-3H-indolium iodide 3**: The product was  
419 synthesized following the *general procedure* with 2-methyl-2H-indazole-3-carbaldehyde **7** (15 mg, 0.1  
420 mmol) and 1-ethyl-2,3,3-trimethyl-3H-indolium iodide **4** (30 mg, 1 eq) (30 mg, dark purple solid, 66%  
421 isolated yield).  $^1\text{H NMR (CD}_3\text{OD, 300 MHz)}$ :  $\delta$  (ppm) = 8.55 – 8.46 (m, 1H), 8.27 – 8.21 (m, 1H), 7.92 –  
422 7.85 (m, 2H), 7.85 – 7.80 (m, 1H), 7.71 – 7.63 (m, 2H), 7.59 – 7.51 (m, 3H), 4.75 (q, 2H,  $J = 7.3$  Hz), 4.54  
423 (s, 3H), 1.93 (s, 6H), 1.67 (t, 3H,  $J = 7.4$  Hz).  $^{13}\text{C NMR (CD}_3\text{OD, 75 MHz)}$ :  $\delta$  (ppm) = 149.8, 145.2, 142.0,  
424 138.41, 138.31, 132.6, 131.0, 130.8, 128.5, 128.3, 124.2, 124.1, 121.5, 120.3, 115.9, 111.2, 53.8, 43.6,  
425 39.8, 26.9, 13.7. **IR**:  $\nu$  ( $\text{cm}^{-1}$ ) = 2976, 2932, 1582, 1569, 1427, 1290, 1243, 1065, 760. **HRMS (ESI<sup>+</sup>)**: found  
426 = 330.1964; calculated  $[\text{M}]^+ = 330.1970$ . **m.p.**: decomposition > 150 °C.

427



428 **2-(2-(1H-indol-3-yl)vinyl)-1-ethyl-3,3-dimethyl-3H-indolium iodide 4:** The product was synthesized  
429 following the *general procedure* with 1H-indole-3-carbaldehyde (145 mg, 1.0 mmol) and 1-ethyl-2,3,3-  
430 trimethyl-3H-indolium iodide (315 mg, 1 eq) (260 mg, pink solid, 59% isolated yield). **<sup>1</sup>H NMR (CD<sub>3</sub>OD,**  
431 **300 MHz):** δ (ppm) = 8.75 (d, 1H, *J* = 15.6 Hz), 8.49 (s, 1H), 8.13 (dd, 1H, *J* = 6.5, 1.8 Hz), 7.73 – 7.37 (m,  
432 7H), 7.27 (d, 1H, *J* = 15.6 Hz), 4.56 (q, 2H, *J* = 7.3 Hz), 1.87 (s, 6H), 1.58 (t, 3H, *J* = 7.3 Hz). **<sup>13</sup>C NMR**  
433 **(CD<sub>3</sub>OD, 75 MHz):** δ (ppm) = 181.4, 151.3, 144.0, 142.2, 141.6, 140.4, 130.3, 129.0, 126.4, 126.0, 124.9,  
434 123.9, 122.0, 118.0, 114.5, 114.2, 105.4, 52.6, 42.0, 27.5, 13.1. **IR:** ν (cm<sup>-1</sup>) = 3398, 3099, 2974, 1585,  
435 1463, 1422, 1298, 1230, 1116, 748. **HRMS (ESI+):** [M]<sup>+</sup> found 315.1866; calculated for [C<sub>22</sub>N<sub>2</sub>3N<sub>2</sub>]<sup>+</sup>  
436 315.1861. **m.p.:** 68-69°C.

437

438 **2-(2-(1methyl-indol-3-yl)vinyl)-1-ethyl-3,3-dimethyl-3H-indolium iodide 5:** The product was  
439 synthesized following the *general procedure* with 1H-indole-3-carbaldehyde (145 mg, 1.0 mmol) and  
440 1-ethyl-2,3,3-trimethyl-3H-indolium iodide (315 mg, 1 eq) (260 mg, pink solid, 59% isolated yield). **<sup>1</sup>H**  
441 **NMR (SO(CD<sub>3</sub>)<sub>2</sub>, 300 MHz):** δ (ppm) = 8.77 (s, 1H), 8.69 (d, *J* = 15.7 Hz, 1H), 8.31 - 8.25 (m, 1H), 7.82 (d, *J* = 7.3  
442 Hz, 1H), 7.77 (d, *J* = 7.7 Hz, 1H), 7.74 - 7.68 (m, 1H), 7.56 (td, *J* = 7.7 Hz, *J* = 1.8 Hz, 1H), 7.51 (d, *J* = 7.7 Hz, 1H),  
443 7.47 - 7.43 (m, 2H), 7.19 (d, *J* = 15.7 Hz, 1H), 4.57 (q, *J* = 7.2 Hz, 2H), 3.99 (s, 3H), 1.81 (s, 6H), 1.44 (t, *J* = 7.2 Hz,  
444 3H). **<sup>13</sup>C NMR (SO(CD<sub>3</sub>)<sub>2</sub>, 75 MHz):** δ (ppm) = 179.2, 148.5, 143.3, 142.7, 140.7, 139.0, 128.9, 127.5, 125.3,  
445 124.5, 123.6, 122.9, 121.3, 114.9, 113.4, 112.1, 104.2, 50.9, 40.6, 34.1, 26.5, 12.8. **HRMS (ESI+):** [M]<sup>+</sup> found  
446 329.2020; calculated for [C<sub>23</sub>N<sub>2</sub>5N<sub>2</sub>]<sup>+</sup> 329.2018.

447

#### 448 **4.2 UV-visible spectroscopy**

449 UV-visible absorption spectra were recorded with a Cary 60 (Agilent) applying a baseline correction.  
450 Steady state fluorescence was measured with a Horiba Fluorolog 3-21 (Hamamatsu photomultiplier  
451 tube R13456) using diluted samples O.D.< 0,1.

452 The dependence of fluorescence upon viscosity was measured by varying the temperature of an  
453 ethylene glycol solution from 10 °C to 60 °C.

454 Fluorescence quantum yields ( $\phi_f$ ) were measured thanks to the equation:  $\phi_f = \phi_{fr} \times [F/F_r] \times [(1-10^{-A})/(1-10^{-A_r})] \times [n/n_r]^2$  where “F” is the integrated fluorescence signal of the dye, “Fr” is the integrated  
455 fluorescence signal of the reference; A and Ar the absorbance the dye and the reference respectively;  
456 n and nr the refractive index of the solvent dye and the solvent reference respectively.

458 Fluorescence lifetimes were measured by TCSPC using a delta diode 506 nm excitation source.

459 J-aggregates emission was measured in an integrating sphere G8 (General Microtechnology and  
460 Photonics) in a Horiba Fluorolog 3-21. J-aggregate were generated by introducing a solution of  
461 10mg/ml of dye inside an integrating sphere capillary then adding the same amount of a saturated  
462 solution of NaCl (6M) or pure water for control experiments. The corrected spectra were measured  
463 and the blank signal was subtracted, taking into account the extinction caused by the sample. Quantum  
464 yields were calculated (excitation 520 nm) using the following equation:  $\phi_f = (f_p - f_s)/(F_1 \times (I_s - I_p))$  where  
465  $f_p$  and  $f_s$  are fluorescence signals (540 - 750nm) with and without product respectively  $F_1$  is the  
466 transmission of a 1% DO filter (Thorlabs) at excitation wavelength and  $I_p$  and  $I_s$  are lamp signal (510 –  
467 530 nm) with and without product respectively.

468

### 469 **3.3 Cell imaging**

470

#### 471 **Cell Culture**

472 As previously described (Raoult et al., 2011), tumoral PC12 cells, derived from a rat adrenal medulla  
473 pheochromocytoma, were cultured in a humidified incubator at 37 °C with an atmosphere of 5 % CO<sub>2</sub>.  
474 Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Illkirch,  
475 France) supplemented with 7 % heat-inactivated fetal bovine serum (Sigma–Aldrich), 7 % horse serum  
476 (Lonza Bioscience, Walkersville, MD, USA), 2.5 % HEPES (4-(2-hydroxyethyl)-1-piperazine

477 ethanesulfonic acid) (Thermo Fisher Scientific), 1 % glutamine (Thermo Fisher Scientific), 100 units/mL  
478 penicillin and 100  $\mu\text{g mL}^{-1}$  streptomycin (Thermo Fisher Scientific).

479  
480 **Cytotoxicity assay**  
481 PC12 cells were prepared for cell viability studies in 24-well plates (ThermoFisher Scientific, Montigny  
482 le Bretonneux, France). The cells were incubated for 1 or 24 hours with a single merocyanine  
483 compound (**1**, **2** or **3**) at a concentration of  $10^{-6}$  M. Two drops of NucGreen™ Dead 488 ReadyProbes  
484 reagent (Thermo Fisher Scientific) per mL of media to test indazole compounds **1**, **2** or **3**, and  $2\mu\text{M}$  of  
485 Ethidium Homodimer (Exc 633nm; Invitrogen) to test **4** and **5** indole compounds (**B**) were added into  
486 wells, followed by further incubation for 10 min at 37 °C. Since the NucGreen and Ethidium are cell-  
487 impermeant and emits green fluorescence when bound to DNA, the viability was determined by  
488 imaging, through transmitted and fluorescence modes, positive cells on an automated boxed inverted  
489 Zeiss CD7 microscope piloted with Zen software (Carl Zeiss, Jena, Germany). Statistical analysis was  
490 performed with the GraphPad Prism 4 software (GraphPad Software Inc.) and a one-way Analysis of  
491 Variance (ANOVA) with a Tukey-Kramer multiple comparisons tests was used.

#### 492 **Cell labelling**

493 For fluorescent labelling with merocyanine compounds, PC12 cells were plated on glass bottom dishes  
494 (grade 1.5 MatTek Corporation, MA, USA) and culture for 1 day. Prior dilution in phenol red-free  
495 Dulbecco's modified Eagle's medium (DMEM), solubilization of compounds was facilitated with the  
496 non-ionic surfactant polyol Pluronic F-127 (ThermoFisher Scientific) (v/v). For live cell imaging, cells  
497 were incubated with indazole and indole merocyanine dyes (final concentration:  $10^{-6}$  M) during 45  
498 min, rinsed once and observed.

499

#### 500 **Confocal microscopy**

501

#### 502 ***Emission/excitation spectra of indazole and indole merocyanine dyes from labelled cells***

503 1-photon excitation and emission spectra were measured using  $\lambda\Delta$  acquisition mode on an inverted  
504 Leica TCS SP5 confocal microscope (Leica Microsystems, Nanterre, France) equipped with a  
505 supercontinuum laser source (NTK photonics, Cologne, Germany) and a resonant scanner (8000 Hz).  
506 Using a 20x objective (0.70, oil immersion), fluorescence emission from labelled living cells was  
507 detected through a hybrid detector (HyD) in the counting mode (Leica Micro-systems, France). In this  
508 configuration, two-dimensional scanning with automatic variations of excitation ( $\Delta$  from 470 to 670  
509 nm, 2 nm step) and emission ( $\lambda$ , from 480 to 795 nm, 10 nm band) was performed and led to a stack  
510 of 1722 images (n=6). Resulting  $\lambda\Delta$  representation and excitation/emission spectra were obtained  
511 using the Excitation Emission Contour Plot of the Leica Application Suite Advanced Fluorescence  
512 software (Leica Microsystems, France).

513

#### 514 ***Subcellular localization of merocyanine dyes in living cells***

515 Subcellular imaging was performed on an inverted Leica TCS SP5 confocal microscope equipped with  
516 a supercontinuum laser source and a conventionnal scanner (400 Hz). In phenol red-free DMEM, **1**, **2**  
517 or **3** indazole merocyanines were excited at 543 nm and fluorescence was collected with a HyD (photon  
518 counting mode) from 560 to 620 nm; **4** or **5** indole merocyanines were excited at 488 nm. A 63x  
519 objective (NA 1.4) and an optical zoom factor of 2.5 were used and z-stacks parameters (z-step = 250  
520 nm; z-stack= between 4 to 6  $\mu$ m) were set. For mitochondria labeling, incubation with Mitotracker  
521 Green TM (Thermo Fisher Scientific) at a concentration of 500 nM during 30 min was performed  
522 following staining with indazole compounds **1**, **2** or **3**. For indole compounds **4** and **5**, Mitotracker Red  
523 TM (Thermo Fisher Scientific) was used. For cell imaging, Mitotracker Green was excited at 488 nm  
524 and fluorescence was collected from 500 to 540 nm, and Mitotracker Red was excited at 594 nm and  
525 fluorescence collected from 605 to 665 nm. All double labeling experiments were performed through  
526 a sequential mode of acquisition to avoid any spectral contamination.

527

#### 528 **Image Analysis**

529 To improve signal-to-noise ratio in confocal images, deconvolution of raw data was obtained through  
530 image processing with Huygens professional 4.5.1 software (SVI, The Netherlands). ImageJ (Rasband,  
531 W.S., U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-  
532 2016) was used to adjust image brightness and contrast and to perform projections of 3D images  
533 (xyz).

534

#### 535 **CRedit authorship contribution statement**

536 **Margot Boujut:** Investigation ; **Arnaud Chevalier :** Conceptualization, Investigation, Writing - Review  
537 & Editing ; **Damien Schapman:** Investigation; **Magalie Bénard:** Investigation; **Ludovic Galas:** Writing -  
538 Original Draft, Visualization, Project administration, Funding acquisition ; **Thibault Gallavardin:**  
539 Conceptualization, Investigation, Writing - Original Draft, Project, administration, Funding acquisition ;  
540 **Xavier Franck :** Writing - Review & Editing, Project administration, Funding acquisition

541

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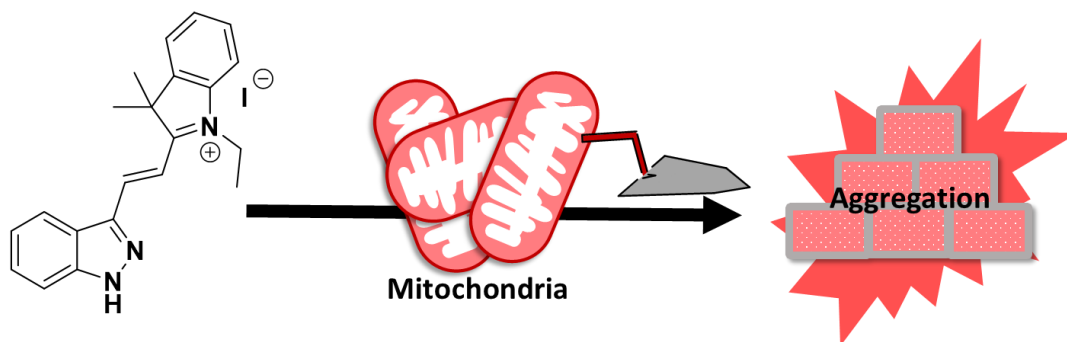
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770 **Graphical abstract**

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