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2 **A dimerization-based fluorogenic dye-aptamer module for RNA imaging in**
3 **live cells**

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16
17 **ABSTRACT**

18 Live-cell imaging of RNA has remained a challenge because of the lack of naturally fluorescent RNAs.
19 Recently developed RNA aptamers that can light-up small fluorogenic dyes could overcome this
20 limitation, but they still suffer from poor brightness and photostability. Here, we propose a concept of
21 cell-permeable fluorogenic dimer of sulforhodamine B dyes (Gemini-561) and corresponding dimerized
22 aptamer (o-Coral) that can drastically enhance performance of the current RNA imaging method. The
23 unprecedented brightness and photostability together with high affinity of this complex allowed, for the
24 first time, direct fluorescence imaging in live mammalian cells of RNA polymerase-III transcription
25 products as well as messenger RNAs labelled with a single copy of the aptamer, i.e. without tag
26 multimerization. The developed fluorogenic module enables fast and sensitive detection of RNA inside
27 live cells, while the proposed design concept opens the route to new generation of ultrabright RNA
28 probes.

32 INTRODUCTION

33 Cells constantly adapt their content to their needs, to changing environment or to pre-determined
34 cell-cycles and differentiation programs by tuning their gene expression landscape. Moreover, live-cell
35 imaging experiments demonstrated that significant cell-to-cell variation in gene expression occurs even
36 within population of isogenic cells within the same environment¹. Currently, imaging of gene expression
37 in live cells relies mainly on proteins genetically modified with either fluorescent proteins² or tags for
38 specific chemical labelings³. Since no naturally fluorescent RNA has been discovered yet, extensive
39 efforts were devoted to the development of RNA imaging technologies⁴.

40 A first breakthrough came with the use of RNA-binding proteins (RBP) fused to fluorescence
41 proteins (FP)^{5,6}. In these completely genetically encoded systems, an array (tens of repeats) of the RNA
42 RBP-binding motif is incorporated into the 3' untranslated region of the target messenger RNA
43 (mRNA). Co-expressing the RBP-FP-coding gene in the same cell allowed for tracking mRNA (upon
44 decoration by FP), which has become a reference method for collecting data on gene expression and
45 RNA trafficking⁷. Substantial simplification is possible by using RNA-based fluorogenic modules⁸ in
46 which bulky FPs are substituted by small fluorogens,⁹ i.e. dyes lighting up their fluorescence upon
47 interaction with a target (bio)molecule¹⁰. In this scheme, the fluorogen is activated upon specific binding
48 to a light-up RNA aptamer¹¹.

49 The capacity of RNA to light-up fluorogenic dyes was first established with an aptamer activating
50 Malachite Green,¹² a dye known for its phototoxicity. Yet, the real breakthrough^{8,13} came with the
51 introduction of the cell-permeable and non-toxic GFP-mimicking fluorogen, 3,5-difluoro-4-
52 hydroxybenzylidene imidazolinone (DFHBI), together with its activating aptamer Spinach¹⁴, later
53 followed by improved aptamers (Spinach²¹⁵, Broccoli¹⁶) and fluorogen derivatives¹⁷. Unfortunately,
54 these systems suffered from limited brightness and photostability compromising the detection of low-
55 abundant RNAs and extended imaging time.¹⁸ Substantial gain in photostability and brightness was
56 achieved using fluorogens based on organic dyes (cyanines¹⁹⁻²² and rhodamines²³⁻²⁵), including those
57 operating by Photoinduced Electron Transfer (PET)²⁶ or Forster Resonance Energy Transfer
58 (FRET)^{20,24,27} mechanisms. For instance, conjugates of sulforhodamine B (SRB) dye with dinitroaniline
59 (DN) PET quencher recover their fluorescence upon association with an aptamer binding the SRB^{24,25,28}

60 (e.g. SRB-2 aptamer) or the DN₂₃ moiety. An alternative strategy, which could significantly improve
61 fluorogen brightness, is to use dye homo-dimers that self-quench in aqueous solution but become
62 fluorescent upon dimer opening after binding to the target biomolecule, a concept that yielded probes
63 for detecting ligand-receptor interaction²⁹ or DNA hybridization³⁰.

64 Brightness and photostability also rely on the aptamer itself as illustrated by Corn^{31,32} pointing the
65 key role of the selection strategy used. Light-up RNA aptamers are usually isolated by Systematic
66 Evolution of Ligand by EXponential enrichment (SELEX)^{33,34}, which allows selecting aptamers with
67 high affinity and selectivity for their target, as exemplified by Mango RNA, a light-up aptamer binding
68 its fluorogen (TO1-biotin) with nM affinity²¹. Yet, since no selection pressure is applied for the
69 fluorogenic capacity, these aptamers often suboptimally activate their fluorogen. This limitation can be
70 overcome by functional screening³⁵⁻³⁷ such as the microfluidic-assisted *in vitro* compartmentalization
71 (μ IVC)³⁸ we recently used to identify brighter mutants of Spinach³⁶ and Mango³⁷.

72 Here, we propose a new concept for preparation of bright and photostable fluorogen for RNA
73 imaging in cells by exploiting dimerization-induced self-quenching of SRB dyes, which yielded the
74 fluorogen Gemini-561. Following a new selection scheme combining SELEX and μ IVC we developed
75 o-Coral, an aptamer of unprecedented compact dimeric structure, able to form a high affinity, bright and
76 photostable complex with Gemini-561, enabling live-cell imaging of mRNAs labelled with a single
77 copy of the aptamer.

78

79 **RESULTS**

80 **Design and synthesis of Gemini-561 fluorogen**

81 We intended to develop a red emitting fluorogen efficiently excited with a common laser (532-561
82 nm), allowing multicolor imaging in combination with eGFP-tagged proteins. Rhodamine fluorophores
83 like SRB fulfill this requirement and possess numerous advantages. First, due to their tendency to form
84 *H*-aggregates³⁹ and their ability to be quenched by different systems (e.g. spirolactamization, PET),
85 rhodamines constitute efficient platforms to develop reliable fluorogenic sensors^{40,41}. SRB bears two
86 sulfonate groups and upon functionalization becomes zwitterionic. Compared to cationic rhodamines or

87 non-charged fluorophores, this feature should increase the polarity of the molecule, thus enhancing its
88 water solubility and preventing non-specific interactions in biological media. Finally, SRB features
89 optimal photophysical properties including elevated quantum yield, good photostability, sharp emission
90 peak and high molar absorption coefficient ($\sim 100,000 \text{ M}^{-1}\cdot\text{cm}^{-1}$). Gemini-561 was designed to promote
91 the dimerization-induced self-quenching of two SRBs. For this purpose, lysine was chosen as a
92 connector to provide a small distance between the dyes and thus ensure efficient π -stacking upon
93 dimerization. Lysine (**1**) and SRB (**2**) derivatives were deprotected and coupled to lead to Gemini-561-
94 alkyne (**3**) (Fig. 1a). The latter was clicked to biotin-PEG-N₃ (the biotin moiety was later used to
95 immobilize the dye during selection experiments) to yield Gemini-561 (**4**).

96 **Spectroscopic properties of Gemini-561**

97 Gemini-561 fluorogenicity was first assessed by spectroscopy. In water, Gemini-561 displayed weak
98 fluorescence intensity with a quantum yield value of 0.01. Moreover, a blue shifted band (530 nm)
99 appeared in the absorption spectrum indicating the formation of dimeric H-aggregate (Fig. 1c), in line
100 with earlier report on the squaraine dimer²⁹. Additionally, excitation spectrum showed that this band did
101 not correspond to emissive specie (Fig. 1d), thus confirming a dimerization-induced quenching
102 phenomenon. However, upon solubilization in methanol, the dimer opened and Gemini-561 displayed
103 absorption and emission spectra similar to free SRB (Fig. 1b to d) along with an impressive increase in
104 the quantum yield value (0.31, Table 1). In a second step, the non-specific opening of the dimer was
105 evaluated, revealing that Gemini-561 conserves its quenched form in various physiological media and
106 is not involved in non-specific interactions with proteins and lipoproteins (Supplementary Fig. 1).
107 Altogether, these experiments demonstrate that Gemini-561 is an effective fluorogenic molecule
108 compatible with biological media, making it a promising candidate for selection of the RNA aptamer.

109 **Isolation of Gemini-561 lighting-up aptamers**

110 We first studied SRB-2 aptamer, reported to specifically interact with sulforhodamine B₂₈ and its
111 derivatives²⁴ However, it was a poor activator of Gemini-561 fluorescence (Fig. 2a, Supplementary Fig.
112 2). We assumed that this weak effect might be attributed to inhibition of dye-aptamer interaction by the
113 dye-dye dimerization.

114 We therefore started an *in vitro* evolution of SRB-2 using a strategy combining SELEX and μ IVC
115 (Fig. 2b, Supplementary Fig. 3) to isolate RNAs endowed with both high affinity and high fluorogenic
116 capacity. SRB-2 mutant library (~ 3.4 mutations per gene) was first generated by error-prone PCR and
117 subjected to a first round of SELEX during which RNAs were challenged to bind bead-immobilized
118 Gemini-561. Upon stringent wash, bound aptamers were recovered and reverse transcribed into cDNAs
119 carrying T7 RNA polymerase promoter. Resulting genes were then subjected to a round of μ IVC
120 screening³⁷. Genes encoding fluorogenic RNAs were finally recovered and used to prime a new round
121 of error-prone PCR. A total of four such evolution cycles (mutagenesis, SELEX, μ IVC) were performed
122 and allowed to gradually improve the average fluorogenic capacity of the population (Fig. 2b), where
123 $\sim 40\%$ of the obtained sequences were significantly more fluorogenic than the parental SRB-2 aptamer.
124 Surprisingly 16 out of the 19 best mutants also displayed a doubled size resulting from complete
125 duplication (dimerization) of SRB-2 sequence upon recombination at a variable position between the 5'
126 and 3' constant regions of two aptamers (Fig. 2c and Supplementary Fig. 4). The exact mechanism of
127 this spontaneous recombination will require a dedicated study. In addition to this duplication, each
128 optimized variant displayed 1 to 6 point mutations concentrated on P1 and P2 regions of the SRB-2
129 (Supplementary Fig. 4), while leaving region J2/3, P3 and L3 largely intact, in agreement with their
130 proposed involvement in SRB recognition²⁸.

131 Among the different clones, 4C10 had a remarkably high activation capacity by forming with
132 Gemini-561 a complex an order of magnitude more fluorescent than with SRB-2 (Fig. 2a). 4C10
133 improvement correlates with a significant increase of affinity ($\sim 73 \pm 1.5$ nM and 441 ± 167 nM for 4C10
134 and SRB-2 respectively, Table 1 and Supplementary Table 1). By further engineering 4C10, we
135 successfully reduced the 19-nucleotide long linker spacing the repeats down to 6 nucleotides while
136 preserving intact fluorogenicity (Fig. 3a). Further reducing this length down to 3 nucleotides made
137 aptamer sensitive to the linker sequence. Therefore, we pursued the study of a 6-nucleotide long linker
138 4C10 derivative, further named "o-Coral" (Supplementary Table 2). The duplication event by itself
139 accounts only partly for the high performances of o-Coral since a molecule made of two wild-type SRB-
140 2 modules displays only 6% of o-Coral fluorescence (Fig. 3b). Progressive reimplantation of o-Coral
141 mutations showed that all the mutations contributed to o-Coral function, with the double mutant

142 U₂₅C/A₃₆G (a mutation found in a third of the 19 best mutants, Supplementary Fig. 4) having the
143 predominant effect. Remarkably, simple introduction of U₂₅C/A₃₆G mutation into SRB-2 did not yield
144 any improvement of the monomer, indicating a synergic effect of the mutation in the dimer (Fig. 3b).
145 Furthermore, U₂₅C/A₃₆G had a higher effect when present in the 5' monomer, whereas introducing it in
146 both monomers did not further improve the aptamer.

147 **o-Coral structural characterization**

148 The role of the RNA module duplication was further studied at a structural level. Enzymatic
149 structural probing characterization⁴² (Supplementary Fig. 5) was in agreement with an overall
150 conservation of the structure initially proposed for the SRB-2 aptamer made of 3 stems and 3 unpaired
151 regions²⁸. Yet, two RNA folding models could account for the probing signal observed: i) a model in
152 which both SRB-2 derived modules fold independently (Supplementary Fig. 6a) and ii) an intertwined
153 folding (Supplementary Fig. 6b). To discriminate both models, we generated a mutant with a
154 destabilized stem (⁶⁷GGUUC₇₁ changed for ⁶⁷CCAAG₇₁, Supplementary Fig. 7) leading to the complete
155 loss of fluorogenic capacity (Fig. 3c). We then tested compensatory mutants relevant either to the
156 independent folding model (²⁰GAACC₂₄ changed for ²⁰CUUGG₂₄) or to the intertwined structure
157 (⁷⁹GGGCC₈₅ changed for ⁷⁹CUUGG₈₅). Interestingly, only the second compensatory mutant rescued o-
158 Coral function (Fig. 3c), suggesting that o-Coral adopts an intertwined folding (Fig. 3e). This model
159 received further support from another mutant (⁶⁹UU₇₀ changed for ⁶⁹CC₇₀) expected to stabilize only the
160 intertwined folding (Supplementary Fig. 8). Finally, it can be noticed that two of the mutations selected
161 during the evolution process (G₁₉A and C₁₃₂U) compensate each other in the intertwined model
162 (Supplementary Fig. 6).

163 In our model, unpaired regions corresponding to J2/3 and L3 in the original SRB-2 aptamer,
164 proposed to form the SRB-binding site²⁸, are preserved (nucleotides 37-43, 49-60, 97-103 and 109-120).
165 The three-dimensional structure of SRB-2 aptamer has not been established yet. However, even though
166 pairs of G bases are found in SRB-2 and o-Coral, the existence of a G-quartet structure, common to
167 many other light-up aptamers⁴³, is unlikely since the complex was found to be insensitive to the nature
168 of the monovalent cation added in the medium (Supplementary Fig. 9a). o-Coral also preserved the low

169 magnesium dependency of SRB-2 aptamer²⁵ by reaching its maximum fluorogenicity at 1 mM
170 magnesium (Supplementary Fig. 9b), a value close to that in cell.

171 **Gemini-561/o-Coral characterization in solution**

172 Among studied dye/aptamer pairs, only combination of Gemini-561 and o-Coral resulted in
173 significant fluorescence enhancement accompanied by disappearance of the short-wavelength band of
174 the non-emissive *H*-aggregate (Supplementary Fig. 10 a-b). Remarkably, a parent analogue of Gemini-
175 561, bearing alkyne group instead of biotin (Gemini-561-alkyne (**3**), Fig. 1a), showed very similar
176 fluorogenic response to o-Coral (Supplementary Fig. 10 c-d), indicating that biotin is not required for
177 the recognition of the aptamer (Supplementary Table 1). Detailed spectroscopic characterization of
178 Gemini-561/o-Coral complex revealed a significant red-shifted absorption and fluorescence emission
179 (by 19 and 16 nm, respectively) compared to those in an “activating” solvent methanol (Fig. 3d and
180 Supplementary Figs. 10 to 12), presumably because of interaction between the dyes and RNA
181 nucleobases. Additionally, we showed that the interaction of Gemini-561 (100 nM) with o-Coral (300
182 nM) was fast, reaching plateau after 400 s (Supplementary Fig. 10e). Interestingly, the quantum yield
183 of the complex reached a higher value than Gemini-561 in MeOH (Table 1), showing that the dye is
184 well confined within the aptamer. Moreover, estimating the brightness (i.e. extinction coefficient ×
185 quantum yield) of the complex indicated that a single copy of o-Coral associated with Gemini-561 is
186 more than 3-fold brighter than eGFP (Table 1), making it the brightest aptamer-based module described
187 so far. Moreover, Fluorescence Correlation Spectroscopy (FCS) confirmed high brightness of Gemini-
188 561/o-Coral complex (at least 1.14-fold brighter than single tetramethyl rhodamine, a close analogue of
189 SRB), suggesting a 1:1 complex composed of a single molecule of Gemini-561 bound to a single copy
190 of o-Coral (Supplementary Table 3). We finally investigated the effect of physiological media on the
191 Gemini-561/o-Coral complex and found that, whereas 10% FBS slightly interferes with the aptamer-
192 fluorogen interaction (Supplementary Fig. 13), SRB-2 aptamer, BSA or DNA did not challenge the
193 fluorescence of the complex, highlighting the compatibility of Gemini-561/o-Coral module with
194 complex cellular environment.

195 **Imaging Gemini-561/o-Coral in live cells**

196 After having ruled out potential cytotoxicity of Gemini-561 (Supplementary Fig. 14), we validated
197 the functionality of Gemini-561/o-Coral complex in live HeLa cells by micro-injecting the preformed
198 complex either directly into their nucleus or into their cytoplasm of living HeLa cells (Supplementary
199 Fig. 15a). In both cases, an intense red fluorescence was readily observed in the presence of the module,
200 whereas the injection of Gemini-561 alone or mixed with SRB-2 aptamer did not yield significant
201 fluorescent signal, confirming the specific activation of Gemini-561 by o-Coral and the suitability of
202 the system for live cell applications. Cell permeability of Gemini-561 was also assessed by incubating
203 HeLa cells with 200 nM fluorogen prior to washing them and microinjecting o-Coral along with
204 Dextran-Alexa-647 conjugate. Both o-Coral and SRB-2 were successfully injected in the cells as
205 attested by Alexa-647 fluorescence, but cytosolic fluorescence of Gemini-561 was observed only in the
206 presence of o-Coral (Supplementary Fig. 15b), demonstrating both cell permeability of the fluorogen
207 and its capacity to detect o-Coral inside live cells.

208 We next evaluated the performances of our system with aptamers synthesized by the cell
209 machinery. o-Coral was inserted into a F30 scaffold⁴⁴ and expressed from a U6 truncated promoter⁴⁵
210 allowing the gene to be transcribed by RNA polymerase III (pol III). The plasmid also contained eGFP-
211 coding region produced from a RNA polymerase II (pol II) promoter and used for identifying transfected
212 green fluorescent cells. Taking advantage of Gemini-561 cell-permeability, we expressed o-Coral gene
213 in live HeLa and HEK293T cells and incubated them with 200 nM Gemini-561 for 5 minutes prior to
214 imaging. Remarkably, green fluorescent transfected cells also displayed intense red (Gemini-561)
215 fluorescence, especially in the nucleus (Fig. 4, Supplementary Fig. 16a) as expected for U6-driven
216 transcripts, confirming that o-Coral preserves RNA sub-cellular localization. Moreover, the sensitivity
217 of this red fluorescence to actinomycin D treatment, a known inhibitor of RNA polymerases, confirmed
218 the requirement of the RNA component. Interestingly, during these live-cell imaging experiments, we
219 noticed cell-to-cell variations in the fluorescence intensity of the Gemini-561/o-Coral (Supplementary
220 Fig. 17), suggesting different transcription states as reported recently with a different aptamer⁹. Taken
221 together, these data show that, owing to elevated brightness and affinity of Gemini-561/o-Coral
222 complex, a single copy of pol III-expressed o-Coral aptamer is sufficient for RNA detection in living
223 mammalian cells.

224 To better understand Gemini-561 cell permeability, we monitored Gemini-561/o-Coral fluorescence in
225 cells and found that internalization kinetics accelerated while increasing Gemini-561 concentration (200
226 vs 50 nM), reaching a plateau in <10 min with both HeLa and HEK293T cells (Supplementary Fig. 18).
227 Yet, further increasing Gemini-561 concentration to 800 nM led to significant background and dye
228 aggregation (Supplementary Fig. 19). Moreover, since Gemini-561 internalization was still observed at
229 4 °C in various cell types (Supplementary Fig. 20), the fluorogen likely reaches the cytosol by direct
230 diffusion through the cell plasma membrane rather than by endocytosis.

231 To extend our technology to Pol II transcripts, we inserted o-Coral/F30 directly into the 3'
232 untranslated region of eGFP gene carried on the plasmid. Excitingly, we found that labelling eGFP
233 mRNA with a single copy of o-Coral was sufficient to detect an intense and homogeneous red
234 fluorescence in eGFP transfected cells (Fig. 4, Supplementary Fig. 16b), while not significantly altering
235 eGFP mRNA level (Supplementary Fig. 21). The mRNA detection with Gemini-561/o-Coral module
236 showed its efficiency in HeLa, HEK293T and U87 cells (Supplementary Fig. 20 and 22), revealing fine
237 difference in mRNA distribution. Indeed, in HEK cells the signal of mRNA was evenly distributed
238 between nucleus and cytosol, similarly to eGFP, whereas in HeLa cells the mRNA signal was slightly
239 stronger in the nucleus (Fig. 4). Additionally, 5S RNA was also successfully imaged in all three studied
240 cell lines (Supplementary Fig. 23), showing universality of our RNA detection approach. It is
241 noteworthy to highlight that, to the best of our knowledge, it is the first time that a mRNA labelled with
242 a single copy of a light-up aptamer can be visualized without the need for extensive image processing⁴⁶.

243 Importantly, the eGFP fluorescence signal correlated well with that of Gemini-561/o-Coral in *egfp*-
244 *3'UTR-o-Coral* expressing HeLa cells (Supplementary Fig. 24). As the protein expression correlates to
245 some extent with corresponding mRNA expression⁴⁷, we concluded that Gemini-561 signal reflects
246 semi-quantitatively the abundance of corresponding mRNA. Interestingly, the same correlation was also
247 observed with cells in which o-Coral and eGFP were produced from genes colocalized on the same
248 plasmid and controlled by constitutive promoters (U6 and CMV, respectively).

249 **Comparison of Gemini-561/o-Coral to other systems**

250 To further demonstrate the advantage of our new fluorogenic system, we systematically compared
251 its performances with other systems using commercially available fluorogens: Broccoli¹⁶, Mango III³⁷
252 and Corn³¹. To properly compare our data with those reported in the literature, aptamers were embedded
253 into the RNA scaffold as they were previously characterized in (i.e. tRNA scaffold for Corn³¹ and F30
254 scaffold for Broccoli³⁷, Mango III³⁷ and o-Coral). Photostability was first assessed in cuvettes where the
255 entire solution of a fluorogenic dye (0.2 μ M) and aptamer (1 μ M) was irradiated with a laser light (Fig.
256 5a). Moreover, to ensure that each system absorbed similar amount of photons, the applied irradiation
257 power density (irradiance) was inversely proportional to the molar absorption coefficient of the
258 corresponding dye at the excitation wavelength used. DFHBI-1T/Broccoli complex showed very poor
259 photostability as its intensity vanished within <1 s, as reported before.^{31,37} Significantly higher
260 photostability was observed for DFHO/Corn and TO1-biotin/Mango III, as their emission changed to
261 much lower extent within 200 s of irradiation, in line with earlier studies.^{31,37} Excitingly, emission
262 intensity of Gemini-561/o-Coral did not show any change in the fluorescence intensity in these
263 conditions, indicating that it is significantly more photostable than the three other systems. Similar
264 experiments performed with RFP and mCherry, also revealed a much higher photostability of Gemini-
265 561/o-Coral (Supplementary Fig. 25), highlighting its advantage over approaches employing FPs (e.g.
266 MS2 system).

267 Second, we compared brightness of the complexes at the single-molecule level using FCS (Mango
268 system was not characterized because of incompatibility with our setup). Single-molecule brightness of
269 DFHO/Corn and DFHBI-1T/Broccoli was correspondingly 0.62 and 0.18 (Supplementary Table 3) with
270 respect to that of reference dye fluorescein (at pH 9), confirming the higher brightness of DFHO/Corn³¹.
271 On the other hand, the single molecule brightness of Gemini-561/o-Coral was 1.14 that of reference dye
272 tetramethyl rhodamine in water. Taking into account that fluorescein and TMR exhibit similar
273 brightness in our FCS setup, the new fluorogenic module, Gemini-561/o-Coral, is ~2-fold brighter than
274 DFHO/Corn in the single molecule experiments. In order to compare brightness of our module with
275 MS2-based technology^{5,6}, we measured FCS for representative fluorescent proteins used in MS2
276 labelling. In comparison to RFP, mCherry and eGFP, our module was respectively 19-, 7- and 7-fold
277 brighter, explaining why our technology does not require multimerization and allows mRNA detection

278 using a single copy of the aptamer tag. Nevertheless, we have to admit that multimerized tandem of 30-
279 copies of these proteins, used in MS2 method, is expected to be brighter than a single copy of our
280 module.

281 Finally, we microinjected the different fluorogen/aptamer complexes into live cells right before
282 taking images (Fig. 5b to d) and measured the signal/background ratio (S/B). Gemini-561/o-Coral
283 system provided images with a S/B value significantly higher than those obtained with other studied
284 systems (Fig. 5b). Image analysis also showed that fluorescence intensity within the cells decayed much
285 slower for Gemini-561/o-Coral (Fig. 5c and d), confirming superior photostability of our system. In an
286 alternative experiment, cells were incubated in the presence of the fluorogen followed by microinjection
287 of the corresponding aptamer. This experiment further confirmed that Gemini-561/o-Coral was
288 significantly more photostable than reference aptamer-based systems (Supplementary Fig. 26), allowing
289 >20 s continuous imaging with only a minor loss of fluorescence intensity. Remarkably, Gemini-561/o-
290 Coral fluorescence can still be detected upon several hours of constant illumination (Supplementary Fig.
291 27).

292

293 **DISCUSSION**

294 Light-up aptamers gained their niche as powerful genetically encoded RNA imaging tools. Due to
295 high modularity of nucleic acids and available SELEX methodologies, a palette of fluorogen-aptamer
296 systems was discovered to shine light on complex cell machinery. Unfortunately, limited brightness and
297 photostability of aptamer-based fluorogenic modules developed to date narrow their broad application.

298 In this work we developed and characterized Gemini-561/o-Coral, a new RNA-based fluorogenic
299 module displaying high brightness, affinity and photostability making it, to the best of our knowledge,
300 the brightest module described so far in the literatures. We reached this goal by combining two key
301 innovations. First, the fluorogen Gemini-561 was designed based on two copies of the bright and
302 photostable sulforhodamine B dye that self-quenches in water and is able to enter the live cells within a
303 few minutes. This quenching mode is important since, upon activation, both fluorophores become
304 strongly fluorescent making such a probe brighter than any monomeric probe described to date. Second,
305 we developed the light-up RNA aptamer o-Coral using an integrated *in vitro* evolution strategy

306 combining rounds of mutagenesis and SELEX in tandem with μ IVC screening. In this scheme, the
307 SELEX step allows isolating RNAs with high affinity for Gemini-561, whereas the μ IVC isolates the
308 most fluorogenic sequences. The obtained o-Coral efficiently opens the self-quenched dimer Gemini-
309 651, thus activating its fluorescence. Using the same overall strategy, it should be possible to further
310 expand RNA imaging toolbox by developing new orthogonal modules made of dimeric (Gemini)
311 fluorophores of any desired color while selecting new specific Coral aptamers.

312 The intertwined dimerized structure of o-Coral may also be advantageous for the future engineering
313 of aptamers. Indeed, both L2 and L2' loops together with the linker region are highly amenable to
314 sequence modification and are attractive sites for inserting other sequences (e.g. sensing aptamers). By
315 doing so, o-Coral could be converted into complex multi-inputs logic gates or biosensors.

316 o-Coral is readily expressed in mammalian cells where it forms a bright complex with Gemini-561
317 that is otherwise not activated by cell components. Similarly to Malachite Green⁴⁸ and DIR2s
318 aptamers⁴⁹, o-Coral does not seem to possess the G-quadruplex organization shared by most of the other
319 structurally characterized light-up aptamers⁴³. This is of particular interest when considering a recent
320 report indicating that most of the RNA G-quadruplex domains would be kept globally unfolded in
321 mammalian cells⁵⁰, suggesting that G-quadruplex-based RNA may not be optimal for being used in
322 living cells.

323 Direct comparison of Gemini-561/o-Coral with the most representative aptamer-based fluorogenic
324 modules in live cells showed clear advantages of the new module in terms of brightness and
325 photostability. Moreover, its significantly higher brightness (7-20-fold according to FCS) and
326 photostability compared to fluorescent proteins explain the capacity to detect RNA using a single copy
327 of the aptamer tag, whereas in case of MS2-eGFP modules^{5,6} a tandem of \sim 30 copies should be used.
328 Imaging of RNA by integrating just a single copy of the aptamer, achieved with Gemini-561/o-Coral,
329 has remained a challenge so far in this technology. However, since dedicated experiments are yet to be
330 performed, it is too early to conclude if the single molecule resolution can be reached without tag
331 multimerization. Overall, Gemini-561/o-Coral system significantly strengthens the toolbox for RNA
332 imaging and shows a new direction in the development of ultrabright fluorogenic aptamer-based
333 modules.

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438

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450

451 **AUTHORS CONTRIBUTIONS**

452 A.S.K., M.C. and M.R. proposed the concept of this study; M.C. synthesized Gemini-561; F.B. and
453 K.T.F. performed main part of experimental work and data analysis with help and supervision of M.R.,
454 M.C., and A.S.K.; F.B. and M.R. developed the aptamer o-Coral, including its analogues, and
455 characterized their structure with the help of S.M; A.A. and F.B. characterized the contribution of the
456 different selected mutations. M.C., K.T.F., F.B. and A.S.K. characterized Gemini-561/o-Coral
457 fluorescence properties in solution; F.B. generated plasmids encoding o-Coral; K.T.F. realized all
458 cellular studies, and, with help of A.S.K. and M.C., he realized all fluorescence imaging of cells.

459

460 **COMPETING INTERESTS**

461 F.B., K.T.F., M.C., A.K., M.R., the University of Strasbourg and the CNRS have filed a patent
462 application covering the technology presented in this manuscript.

463

464

465 **ONLINE METHODS**

466 **Synthetic procedures**

467 Synthetic procedures of Gemini-561-alkyne (**3**) and Gemini-561 (**4**) are given as a Supplementary
468 Note available online.

469

470 **Optical Spectroscopy**

471 Optical spectroscopy was performed with Milli-Q water (Millipore®). All the solvents were
472 spectroscopic grade. Absorption and emission spectra were recorded on a Cary 4000 Scan ultraviolet–
473 visible spectrophotometer (Varian) and a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon)
474 equipped with a thermostated cell compartment, respectively. For standard recording of fluorescence
475 spectra, the emission was collected 10 nm after the excitation wavelength. All the spectra were corrected
476 from wavelength-dependent response of the detector and measured at room temperature (25 °C).
477 Absorbance values of all solutions were systematically below 0.1 at the maximum. Quantum yields were
478 determined using a reference dye (Rhodamine B in water).

479 **Gene library generation**

480 The sequence coding for the SRB aptamer²⁸ was flanked with constant regions at 5'
481 (GGGAGACAGCTAGAGTAC) and 3' (GACACGAGCACAGTGTAC) ends to allow DNA
482 amplification and RNA reverse transcription. Mutant libraries were generated by error prone polymerase
483 chain reaction (PCR) by subjecting 10 fmoles of DNA (diluted in 200 µg/ml of yeast total RNA solution
484 (Ambion)) to 4 amplification cycles in the presence of Fwd
485 (CTTTAATACGACTCACTATAGGGAGACAGCTAGAGTAC) and Rev
486 (GACACGAGCACAGTGTAC) primers as well as nucleotide analogues (JBS dNTPMutagenesis Kit,
487 Jena Bioscience) as described before³⁸. 1 ng of PCR products was amplified in a second PCR mixture
488 containing 10 pmoles of each primer (Fwd and Rev), 0.2 mM of each dNTPs, 5 U of PhireII®
489 (Fermentas) and the corresponding buffer (Fermentas). The mixture was thermocycled starting with an
490 initial step of denaturation of 30 sec at 95°C followed by 25 cycles of: 5 sec at 95°C and 30 sec at 60°C.
491 The PCR products were purified following the "Wizard® SV Gel and PCR Clean-up System" (Promega)
492 kit instructions and the quantity of DNA was determined by NanoDrop™ measurement.

493 ***In vitro* transcription and RNA purification**

494 Genes coding for aptamers were PCR amplified with the same procedure used before (25 cycles of
495 PCR using PhireII enzyme). 1 µg of PCR products was then *in vitro* transcribed in 500 µl of mixture
496 containing 2 mM of each NTP (Larova), 25 mM MgCl₂, 44 mM Tris-HCl pH 8.0 (at 25°C), 5 mM DTT,
497 1 mM Spermidine and 17.5 µg/ml T7 RNA polymerase (prepared in the laboratory). After 3h of
498 incubation at 37°C, 1000 units of Baseline-Zero™ DNase (Epicentre) and the corresponding buffer were
499 added to the mixture and a second incubation of 1 h at 37°C was performed. RNA was then recovered
500 by phenol extraction. *In vitro* transcribed RNA was purified using ion exchange chromatography
501 (FastFlow DEAE sepharose, GE Healthcare) by loading the RNA in and washing the resin with
502 bind/wash buffer (50 mM NaCl, 50 mM Tris-HCl pH 7.5 and 10% Glycerol) and eluting it with elution
503 buffer (600 mM NaCl and 50 mM Tris-HCl pH 7.5). Alternatively, RNA was gel purified by ethanol
504 precipitating transcription mixture and dissolving the pellet into loading buffer (0.05% bromophenol
505 blue, 20% glycerol, TBE 1x, 8M urea). The solution was then loaded on a 12% denaturing 8 M urea
506 acrylamide/bisacrylamide gel. The piece of gel containing RNA was identified by UV shadowing, and
507 the RNA electroeluted as described before³⁸. Eluted RNA was then ethanol precipitated, the washed
508 pellets were dissolved in DEPC treated water and quantified with Nanodrop (Thermo Scientific).

509 **SELEX**

510 100 µL of streptavidin-agarose beads (Sigma-Aldrich) were washed with 200 µL of activation
511 buffer (100 mM NaOH, 50 mM NaCl). The beads were then centrifuged 5 minutes at 5000 g and room
512 temperature and the supernatant was removed by pipetting. This procedure was repeated with 200 µL
513 of pre-wash buffer (40 mM potassium phosphate buffer pH 7.5, 100 mM KCl, 1 mM MgCl₂ and 0.05%
514 Tween 20) and finally 200 µL of wash buffer (pre-wash buffer supplemented with 1 mg/mL BSA (New
515 England Biolabs), 0.1 mg/mL sodium heparin (Sigma-Aldrich) and 200 µg/ml yeast total RNA
516 (Ambion). The resin was loaded into a cartridge (Plastic small column CS-20 ABT) previously
517 equilibrated by an overnight incubation with wash buffer at 4°C. Then, 500 µL of wash buffer
518 supplemented with 10 nmoles of biotinylated Gemini-561 dye were added on the beads at a controlled
519 flow-rate of 10 mL/h using a syringe pump (PhD 2000, Harvard Apparatus). Afterward, the unbound

520 fluorophore was washed away by 20 mL of wash buffer (20 mL / h). ~ 50 µg of purified RNA were
521 introduced in 250 µL of ultra-pure DEPC-treated water and renatured by 2 min at 85°C followed by 5
522 min at 25°C. Then, 250 µL of twice concentrated washing buffer were added and the mixture was
523 infused through the Gemini-561 substituted resin at a flow-rate of 1.5 mL/h. Unbound RNAs were
524 eliminated per 20 mL of wash buffer infused at 20 mL/h. This initial wash was followed by three
525 additional washes of 15 mL of buffer while reducing the ionic strength (respectively 100 mM, 10 mM
526 and 1 mM KCl). The selection pressure was further increased during the last round by introducing 5 µM
527 of free Gemini-561 dye during the last wash of the column. The beads were then collected using a
528 Pasteur pipette, centrifuged and placed in 100 µL of elution buffer (95% formamide and 25 mM EDTA).
529 After 2 minutes of heating at 90°C, the beads were centrifuged, the supernatant was recovered and the
530 RNA precipitated as above. RNA was pelleted, washed and resuspended in 50 µL of 2 µM Rev primer
531 solution. The mixture was heated for 2 min at 85°C and cooled at 25°C for 5 min. 50 µL of reaction
532 mixture containing 0.5 mM of each dNTP, 400 U of reverse transcriptase (Maxima H Minus,
533 ThermoFisher) and the corresponding 2x concentrated buffer were added and the mixture and incubated
534 1h00 at 55°C. The resulting cDNA was then extracted with a mixture Phenol / Chloroform / Isoamyl
535 alcohol 25/24/1 (Roth) and precipitated. cDNA was recovered by 30 minutes of centrifugation at 21000
536 g and 4°C, washed, dried, resuspended in 250 µL of PhireII PCR mixture and amplified by PCR as
537 described above.

538 **Droplet-based microfluidics**

539 Microfluidic chips were made of polydimethylsiloxane (PDMS) as described before³⁸.
540 *i. Droplet digital PCR.* DNA mutant libraries were diluted in 200 µg/mL yeast total RNA solution
541 (Ambion) down to ≈ 8 template DNA molecules per picoliter. 1 µL of this dilution was then introduced
542 in 100 µL of PCR mixture containing 0.2 µM of each primer (Fwd and Rev), 0.2 mM of each dNTPs,
543 20 µM of coumarin, 0.1% Pluronic F68 (Sigma), 5 U of PhireII enzyme (Fermentas) and the
544 corresponding buffer (Fermentas). The mixture was loaded in a length of PTFE tubing and infused into
545 droplet generator microfluidic chip where it was dispersed in 2.5 pL droplets (production rate of ≈12
546 000 droplets/s) carried by Novec 7500 fluorinated oil (3M) supplemented with 3% of a fluorosurfactant

547 (proprietary synthesis). Droplet production frequency was monitored in real time using an optical device
548 and software developed by the team³⁸ and used to determined droplet volume. 2.5 pL droplets were
549 generated by adjusting pumps flowrates (MFCS, Fluigent). The emulsion was collected in 0.2 mL tubes
550 and subjected to an initial denaturation step of 2 min at 98°C followed by 30 cycles of: 10 sec at 98°C,
551 30 sec at 55°C, 30 sec at 72°C. Droplets were then reinjected into a droplet fusion microfluidic device.

552 *ii. Droplet fusion.* PCR droplets were reinjected and spaced into a fusion device at a rate of ~1500
553 droplets/s. Each PCR droplet was then synchronized with a 16 pL *in vitro* transcription (IVT) droplet
554 containing 2 mM each NTP (Larova), 25 mM MgCl₂, 44 mM Tris-HCl pH 8.0 (at 25°C), 5 mM DTT,
555 1 mM Spermidine, 0.1% of Pluronic F68 (Sigma), 1 µg of pyrophosphatase (Roche), 500 nM Gemini-
556 561, 1 µM coumarin acetate (Sigma) and 17.5 µg/mL T7 RNA polymerase (prepared in the laboratory).
557 IVT mixture was loaded in a length of PTFE tubing and kept on ice during all experiment. PCR droplets
558 were spaced and IVT droplets produced using a single stream of Novec 7500 fluorinated oil (3M)
559 supplemented with 2% (w/w) of fluorinated. Flow-rates (MFCS, Fluigent) were adjusted to generate 16
560 pL IVT droplets and maximize synchronization of 1 PCR droplet with 1 IVT droplet. Pairs of droplets
561 were then fused with an AC field (400 V at 30 kHz) and the resulting emulsion was collected off-chip
562 and incubated for 2 h at 37°C.

563 *iii. Droplet analysis and sort.* The emulsion was finally reinjected into an analysis and sorting
564 microfluidic device at a frequency of ~ 150 droplets/s and spaced with a stream of surfactant-free Novec
565 7500 fluorinated oil (3M). The orange fluorescence (Gemini-561 in complex with the aptamer) of each
566 droplet was analyzed and the 1–2% most orange fluorescence droplets were sorted. The gated droplets
567 were deflected into collecting channel by applying an AC fields (1000 V 30 kHz) and collected into a
568 1.5 mL tube. Sorted droplets were recovered from the collection tubing by flushing 200 µL of Novec
569 fluorinated oil (3M). 100 µL of 1H, 1H, 2H, 2H-perfluoro-1-octanol (Sigma-Aldrich) and 200 µL of
570 200 µg/mL yeast total RNA solution (Ambion) were then added and the droplets broken by vortexing
571 the mixture. DNA-containing aqueous phase was recovered, and the DNA recovered by PCR.

572 **TA cloning**

573 The DNA contained in the libraries obtained after the last two rounds of μ IVC screening were PCR-
574 amplified as described above but using the DreamTaq® enzyme and buffer (Fermentas) instead of
575 PhireII (Fermentas). PCR products were purified ("Wizard® SV Gel and PCR Clean-Up System" kit
576 (Promega)) and inserted into the cloning vector of the "insTAclone PCR Cloning" kit (Thermo-
577 Scientific) following the manufacturer's recommendations by overnight ligation at 4°C. ElectroTEN
578 Blue bacteria (Agilent) were transformed by electroporation and plated onto a 2YT / agar / Ampicillin
579 (100 μ g / mL) plate.

580 Individual colonies were used to seed 20 μ L of PhireII PCR mixture (see above) while the rest of
581 the colony was introduced in 3 mL of liquid medium 2YT / Ampicillin (100 μ g / ml) overnight at 37°C
582 under agitation. Upon thermocycling, 2 μ L of PCR product were added to 18 μ L of *in vitro* transcription
583 mixture (see above) supplemented with 100 nM of Gemini-561. The mixture was then incubated at 37
584 °C in a thermocycler (Stratagene Mx3005P, Agilent Technologies) and the fluorescence (ex/em 575/602
585 nm) was monitored for 2 hours. Finally, plasmid DNA was extracted from bacteria of interest using
586 "GenElute Plasmid Miniprep" kit (ThermoFisher) and sequenced by the Sanger method (GATC
587 Biotech).

588 **Real-time IVT measurements**

589 PCR products of each selection cycle were purified ("Wizard® SV Gel and PCR Clean-up System"
590 (Promega)) kit and quantified (NanoDrop™). 50 ng of pure DNA was introduced into 38 μ L of *in vitro*
591 transcription mixture (see above) supplemented with 100 nM of Gemini-561. This mixture was then
592 incubated at 37 ° C in a real-time thermocycler (Stratagene Mx3005P, Agilent Technologies) and the
593 fluorescence was monitored as above.

594 **Fluorescence measurement on purified RNA**

595 1 μ M purified RNA was heated for 1 min at 90 °C and cooled at 4°C for 1 min. The solution was
596 then supplemented with 1 volume of a twice concentrated mixture containing 80 mM potassium
597 phosphate buffer pH 7.5, 2 mM MgCl₂, 0.1% Tween 20, 200 mM of salt (KCl, NaCl, LiCl or CsCl) and
598 100 nM Gemini-561. The mixture was then incubated for 10 min at 25°C prior to measuring the

599 fluorescence at 25°C on a real-time thermocycler (ex/em 575/602 nm, Mx 3005P, Agilent) or on
600 microplate reader (ex/em 560/600 nm, SpectraMax iD3, Molecular Devices).

601

602 **RNA probing**

603 20 µg of RNA were first dephosphorylated for 20 min at 37 °C using 1 U FastAP (Fermentas) per
604 µg of RNA. Upon phenol/chlorophorm extraction and RNA precipitation, dephosphorylated RNA was
605 5' labelled by incubating 5 µg of dephosphorylated RNA with 50 µCi of [P32]γATP and 10 U of T4
606 polynucleotide kinase, with T4 PNK 1× buffer in final volume of 15 µL during 1h00 at 37 °C prior to
607 be phenol/chloroform extracted, precipitated, pelleted. Labeled RNA was then gel-purified and eluted
608 from the gel by an overnight incubation at 4 °C and gently mixing in RNA Elution Buffer (500 mM of
609 ammonium acetate and 1 mM of EDTA). Eluted radiolabeled RNA was extracted by phenol/chloroform
610 treatment, precipitated in ethanol and pelleted as described above. The RNA is resuspended in nuclease-
611 free water. The specific activity (cpm/µL) was calculated by measuring 1 µL in a radioactivity counter
612 “Multi-Purpose Scintilliator Counter” (Beckman) by Cerenkov counting. Labeled RNA (200,000 cpm)
613 was renatured by heating it for 1 min at 90°C then 1 min on ice and then pre-incubated at 20 °C for 15
614 min in a buffer containing 20 mM of this-HCl pH7.5, 1mM of MgCl₂ and 150 mM of KCl. 1 µg of total
615 RNA was then added to the preparation and RNAs were incubated with T1 enzyme (0.25 U, 0.5 U, 1
616 U), T2 enzyme (0.0125 U, 0.025 U, 0.05 U) and V1 enzyme (0.001 U, 0.002 U, 0.004 U) for 5 min at
617 20°C (T1 and V1) or 10 min at 20°C (T2) or water (Ctrl lane). The same amount of digested product
618 was loaded on a 10% denaturing 8 M urea acrylamide/bisacrylamide gel in parallel to an alkaline
619 hydrolysis ladder and a denaturing T1 as described previously⁴². The radiolabeled RNAs were then
620 visualized on autoradiographic film.

621

622 **Affinity measurements**

623 To measure K_D , the concentration of renatured and purified RNAs was progressively increased from
624 2.45 nM to 40 µ M for SRB-2 and from 3.9 nM to 4 µM for 4C10 and o-Coral aptamer with 100 nM
625 (for SRB-2) or 25 nM (for 4C10 and o-Coral) of Gemini-561 in 40 mM potassium phosphate buffer pH

626 7.5, 100 mM KCl, 1 mM MgCl₂ and 0.05% Tween 20. The fluorescence was measured on a microplate
627 reader (ex/em 580/620 nm, SpectraMax iD3, Molecular Devices).

628

629 **Expression vectors design**

630 The sequences coding for o-Coral or 20 nucleotides from Broccoli aptamer (Ctrl) were introduced
631 downstream a U6 promotor into a F30-scaffold (Supplementary Table 2) contained into a pUC57 vector
632 (Proteogenix) *via* a restriction (SbfI) / ligation step. The entire sequences (pU6_o-Coral_F30 or
633 pU6_Ctrl_F30) were then introduced into an eGFP-N1 vector (Clontech) using AflIII restriction sites.
634 Alternatively, o-Coral-F30 or Ctrl-F30 sequences were introduced directly in the 3'UTR of the eGFP
635 coding sequence under the control of a CMV promotor by a restriction (MfeI) / ligation step.

636

637 **RNA extraction and RT qPCR**

638 Total RNA was extracted from HeLa cells (transfected with plasmid carrying *gfp* of *gfp-o-Coral*
639 genes) using Monarch[®] Total RNA Miniprep kit (New England Biolabs) according to manufacturer's
640 instructions. 4 µg of total RNA were used for DNase digestion using dsDNase enzyme (Thermo
641 Scientific). cDNA was then synthesized using Maxima minus H reverse transcriptase (Thermo
642 Scientific) following manufacturer's instructions and specific cDNAs were then quantified by qPCR: 1
643 µL of cDNA was introduced into 19 µL of Ssofast evagreen qPCR mixture (Biorad) supplemented in
644 eGFP specific primers or into 19 µL of Ssoadvanced qPCR mixture (Biorad) supplemented with S18 or
645 GAPDH specific primers and Taqman probes (Biorad). Real-time PCR was carried out on an CFX96
646 Touch[™] System (Biorad) starting with an initial denaturation step of 2 min at 95°C followed by 40
647 cycles of: 5 sec at 95°C and 30 sec at 60°C. Reactions were run in triplicate in three independent
648 experiments. The housekeeping genes GAPDH and S18 were used as internal controls to normalize the
649 variability in expression levels.

650

651 **Cell culture and transfection**

652 HeLa (ATCC[®] CCL-2[™]) and HEK293T (ATCC[®] CRL-3216[™]) cells were grown in Dulbecco's
653 Modified Eagle Medium without phenol red (DMEM, Gibco-Invitrogen) supplemented with 10% fetal

654 bovine serum (FBS, Lonza), 1% L-Glutamine (Sigma Aldrich) and 1% antibiotic solution (Penicillin-
655 Streptomycin, Sigma-Aldrich) at 37°C in humidified atmosphere containing 5% CO₂. U87MG (ATCC®
656 HTB-14™) were grown in Minimum Essential Medium (MEM, Gibco-Invitrogen) supplemented with
657 10% FBS, 1% Ultra-Glutamine (Gibco-Invitrogen) and 1% antibiotic solution. RNA-coding constructs
658 were transfected directly into a 35 mm glass-bottomed imaging dish (IBiDi®) using FuGene HD
659 (Promega) transfecting agent following recommended manufacturer protocol. Imaging experiments
660 were performed 16-48 h post-transfection.

661

662 **Cellular Imaging**

663 Cells were seeded onto a 35 mm glass-bottomed imaging dish (IBiDi®) at a density of 3-5×10⁴
664 cells/well 48 h before the microscopy measurement. 16-24 h prior to imaging, cells were transfected
665 with corresponding DNA plasmid. For imaging, the culture medium was removed and the attached cells
666 were washed with Opti-MEM (Gibco-Invitrogen). Next, the cells were incubated in Opti-MEM with
667 Hoechst (5 µg/mL) to stain the nuclei and in the presence of Gemini-561 dye (0.2 µM) for 5 min, the
668 living cells were washed twice with Opti-MEM and visualized in Opti-MEM or were fixed in 4% PFA
669 in PBS for 5 minutes before being washed twice in PBS. The images were acquired in epi-fluorescence
670 mode with a Nikon Ti-E inverted microscope, equipped with CFI Plan Apo × 60 oil (NA = 1.4)
671 objective, and a Hamamatsu Orca Flash 4 sCMOS camera. The acquisition settings were: Hoechst (ex.
672 395 nm, em. 510±42 nm), eGFP (ex: 470 nm, em: 531±40 nm), G561/o-Coral complex (ex: 550 nm,
673 em: 595±40 nm) and Alexa-647 (ex: 638 nm, em: LP 647 nm). The images were recorded using NIS
674 Elements and then processed with Icy software.

675

676 **Microinjection experiments**

677 Cells were seeded onto a 35 mm glass-bottomed imaging dish (IBiDi®) at a density of 1×10⁵
678 cells/well 24 h before the microscopy measurement. For imaging, the culture medium was removed and
679 the attached cells were washed with Opti-MEM (Gibco-Invitrogen). Next, the cells were incubated in
680 Opti-MEM with Hoechst (5 µg/mL) to stain the nuclei. *In vitro* transcribed and purified aptamers were

681 preincubated with respective fluorogen for 10 min in selection buffer to form complex at corresponding
682 concentrations indicated in figures. Microinjection parameters: $P_i=90$ [hPa]; $T_i=0.3$ [s]; $P_c=10$ [hPa].
683 The images were acquired in epi-fluorescence mode with a Nikon Ti-E inverted microscope, equipped
684 with CFI Plan Apo $\times 60$ oil (NA = 1.4) objective, and a Hamamatsu Orca Flash 4 sCMOS camera. The
685 acquisition settings were: Hoechst (ex. 395 nm, em. 475 ± 50 nm), Broccoli (ex: 470 nm, em: 531 ± 40
686 nm); Corn (ex: 470 nm, em: 531 ± 40 nm); Mango (ex: 470 nm, em: 531 ± 40 nm); Coral (ex: 550 nm,
687 em: 595 ± 40 nm). The images were recorded using NIS Elements and then processed with Icy software.

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689 **DATA AVAILABILITY STATEMENT**

690 The data supporting the findings of this study are available within the paper and its supplementary
691 information file.

692

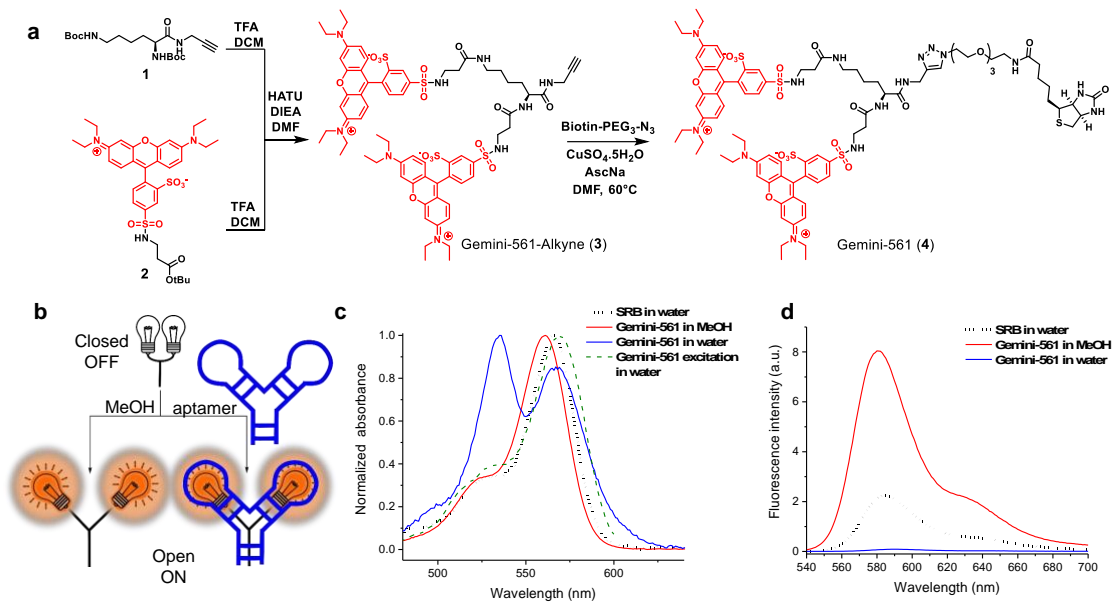
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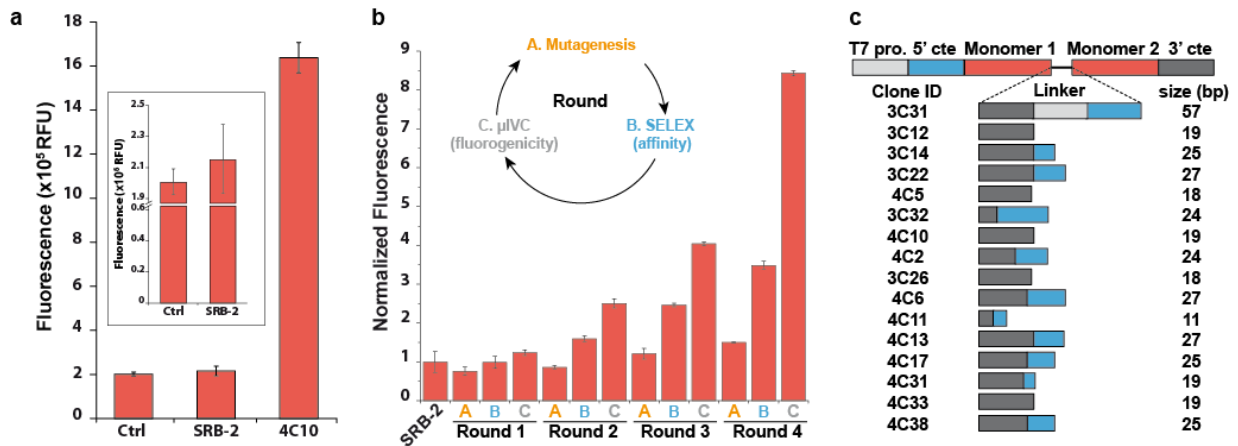
695 **Table 1 | Spectral and biochemical properties of Gemini-561 alone or in complex with SRB-2 or**
 696 **o-Coral aptamers.**
 697

	$\lambda_{\text{Abs max}}$ (nm)	ϵ (M ⁻¹ .cm ⁻¹)	$\lambda_{\text{Em max}}$ (nm)	Q.Y.	Fluorescence Enhancement	K_D (nM)	Relative Brightness
eGFP	490	39,200	508	0.68	/	/	1.00
Buffer	535	100,000	590	0.02	1.0	/	0.08
MeOH	561	162,000	580	0.31	10.3	/	1.88
SRB-2	567	98,000	597	0.04	1.2	441 ± 167.5	0.15
o-Coral	580	141,000	596	0.58	12.8	73 ± 1.5	3.07

698 Measures were performed in selection buffer (40 mM phosphate buffer pH7.5, 100 mM KCl, 1mM MgCl₂
 699 and 0.05% Tween-20). Fluorescence quantum yield (QY) and enhancement were obtained at an excitation
 700 wavelength of 530 nm. K_D values are the mean of n = 3 independent experiments ± 1 SD.



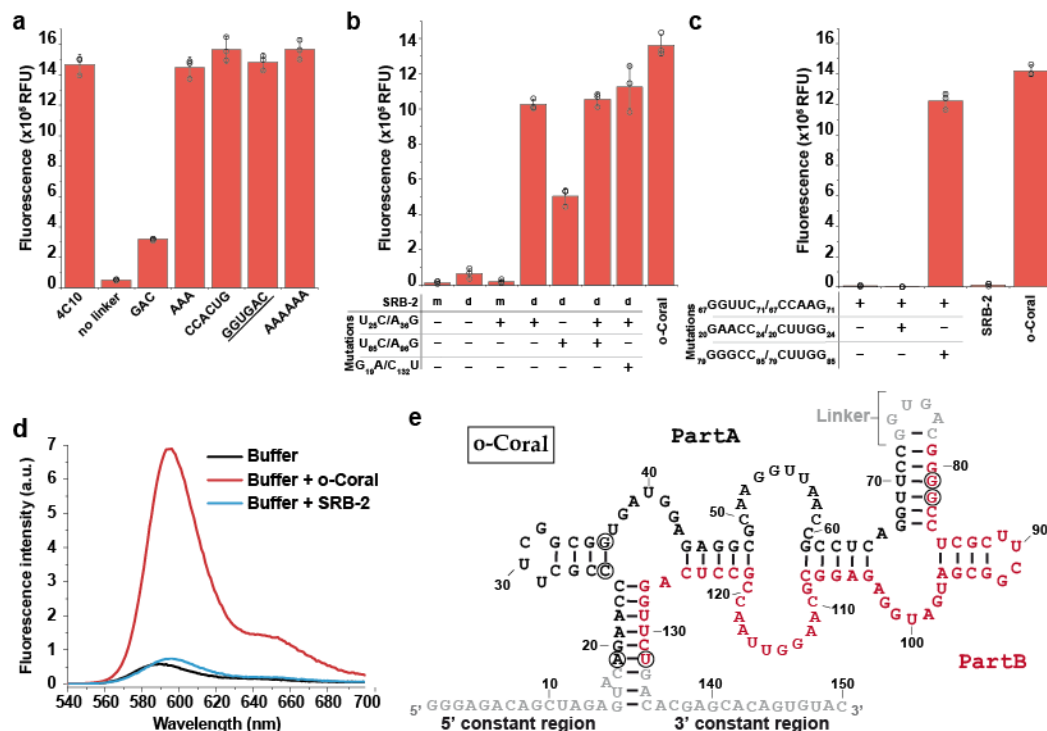
701
 702 **Figure 1 | Design, synthesis and fluorogenicity of Gemini-561.** (a) Synthesis of Gemini-561. (b)
 703 Concept of fluorogenic response of Gemini-561 to environment (organic solvent) and aptamer. (c)
 704 Absorption and excitation spectra of Gemini-561 (200 nM) in water and methanol and SRB (200 nM)
 705 in water. Results were found identical in n = 3 independent experiments. (d) Fluorescence spectra of
 706 Gemini-561(200 nM) in water and methanol and SRB (200 nM) in water. Results were found similar in
 707 n = 3 independent experiments.
 708
 709



711 **Figure 2 | Isolation of Gemini-561 lighting-up aptamers by *in vitro* evolution.** (a) Gemini-561
 712 activation capacity of the parental SRB-2 and the evolved 4C10 variant. 500 nM of RNAs were
 713 incubated with 50 nM of Gemini-561 and the fluorescence was measured at λ ex/em = 560/600 nm. The
 714 values are the mean of n = 3 independent experiments and the error bars correspond to \pm 1 standard
 715 deviation. (b) Monitoring of the evolution process. For each round, the enriched library was transcribed
 716 *in vitro* in the presence of 100 nM of Gemini-561 and the fluorescence monitored. The fluorescence
 717 apparition rate was computed for each library and normalized to that of the parental SRB-2 aptamer.
 718 The inset schematizes the different steps (A, B and C) of an evolution round. The values are the mean
 719 of n = 3 independent experiments, each measurement being shown as an open circle. The error bars
 720 correspond to \pm 1 standard deviation. (c) Schematic representation of genes coding for the 16 dimerized
 721 variants found among the 19 best aptamers at the end of the evolution process. For each variant, the
 722 width and the color of the box respectively inform on linker length (numerical value given on the right)
 723 and the nature of the sequence (light gray: T7 promoter, blue: 5'constant, dark grey: 3' constant). Red
 724 boxes correspond to SRB-2-derived core. The clone ID refers to the round of selection from which the
 725 clone was extracted (first number) and the clone number assigned during the final screening.

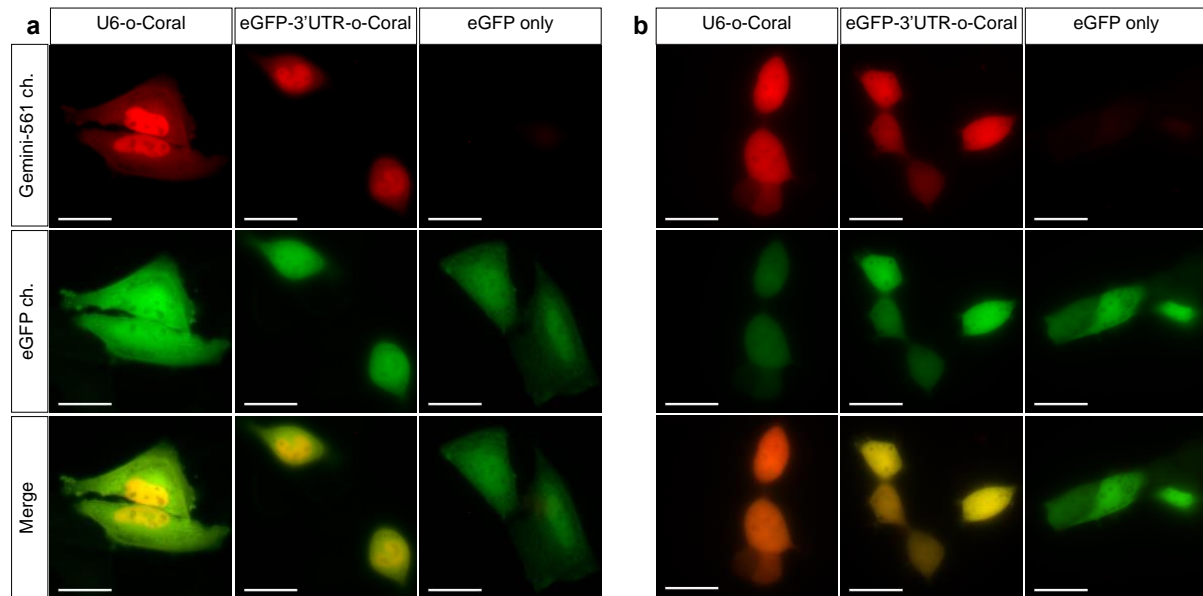
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730 **Figure 3 | Characterization and engineering of the evolved molecule.** (a) Impact of linker size and
 731 sequence on the capacity of 4C10 aptamer to activate Gemini-561 fluorescence. 500 nM of RNAs were
 732 incubated with 50 nM of Gemini-561 and the fluorescence was measured at $\lambda_{ex/em} = 560/600$ nm. The
 733 underlined sequence corresponds to o-Coral linker. (b) Contribution of the dimerization and the
 734 mutations to o-Coral functionality. SRB-2 aptamer was used as scaffold either in its monomeric (m) or
 735 dimeric (d) form containing o-Coral linker. Indicated mutations were then implemented and the different
 736 constructs tested as above. (c) Identification of interacting regions. A destabilized mutant
 737 (${}_{67}\text{GGUUC}_{71}/{}_{67}\text{CCAAG}_{71}$) of o-Coral and two potential compensatory mutants (1:
 738 ${}_{67}\text{GGUUC}_{71}/{}_{67}\text{CCAAG}_{71-20}\text{GAACC}_{24}/{}_{20}\text{CUUGG}_{24}$ and 2:
 739 ${}_{67}\text{GGUUC}_{71}/{}_{67}\text{CCAAG}_{71-79}\text{GGGCC}_{85}/{}_{79}\text{CUUGG}_{85}$) were prepared and tested as above. The values (a-c)
 740 are the mean of $n = 3$ independent experiments, each measurement being shown as an open circle. The
 741 error bars correspond to ± 1 standard deviation. (d) Fluorescence emission spectra of Gemini-561 (200
 742 nM) in absence and in the presence of RNA aptamers (600 nM). Excitation wavelength was 530 nm.
 743 Results were found similar in $n = 3$ independent experiments. (e) Model of secondary structure for o-
 744 Coral aptamer. This model was established based on enzymatic probing experiments (**Supplementary**
 745 **Fig. 8**) and mutagenesis experiments shown on c. SRB-2 derived sequences (Part A and B) are shown
 746 in black or red whereas the constant regions and the linker are shown in grey. Acquired mutations found
 747 to contribute to o-Coral function are circled in black.

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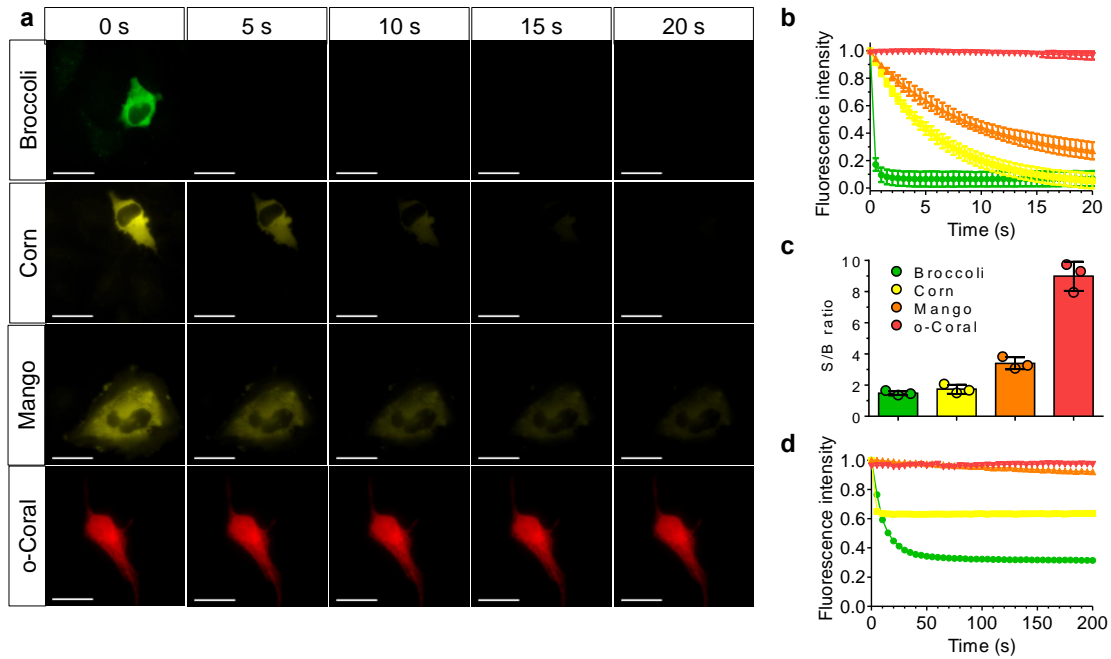


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753 **Figure 4 | Live-cell imaging of o-Coral expressed from pol. II and pol. III promoter.** Live cell
 754 imaging of HeLa (a) and HEK293T (b) cells expressing U6-o-Coral, the *egfp* mRNA eGFP-3'UTR-o-
 755 Coral, or eGFP only. Cells were incubated with Gemini-561 (200 nM) for 5 min before imaging.
 756 Hoechst was used to stain the nucleus (5 μ g/mL). The images were acquired using a 500 ms exposure
 757 time. Gemini-561 in red (ex: 550 nm, em: 595 \pm 40 nm) and eGFP in green (ex: 470 nm, em: 531 \pm 40
 758 nm). Results were found similar in n = 3 independent experiments. Scale bar is 30 μ m.

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762 **Figure 5 | Comparative analysis of photostability by fluorescence microscopy and spectroscopy.**
763 (a) Photostability of G561/o-Coral (0.2 μ M/1 μ M) compared to Broccoli+DFHBI-1T (0.2 μ M/1 μ M),
764 Corn+DFHO (0.2 μ M/1 μ M), Mango + TO1-Biotin (0.2 μ M/1 μ M). Each complex was excited at the
765 same molar extinction coefficient value: 30,000 M⁻¹ cm⁻¹. Broccoli, Corn and Mango were excited using
766 488 nm laser (7.75 mW cm⁻², 11 mW cm⁻², 10 mW cm⁻² respectively) and o-Coral was excited using
767 532 nm laser (7 mW cm⁻²). Fluorescence intensity was monitored at 507 nm for Broccoli, 545 nm for
768 Corn, 535 nm for Mango and 596 nm for o-Coral. (b-d) Photostability and signal to background
769 ratio measurement in live HeLa cells. *In vitro* transcribed and purified aptamers were preincubated with
770 respective fluorogenic dyes for 10 min in selection buffer to form complex. Complexes were
771 microinjected in live HeLa cells using 5 μ M dye and 20 μ M aptamer concentration. Microinjection
772 parameters: Pi=90 [hPa]; Ti=0.3 [s]; Pc=10 [hPa]. Consecutive images were acquired, each using a 500
773 ms exposure time. The excitation power was adjusted for the fluoromolecules to absorb similar amount
774 of photons. Broccoli (ex: 470 nm, em: 475 \pm 50 nm); Corn (ex: 470 nm, em: 531 \pm 40 nm); Mango (ex:
775 470 nm, em: 531 \pm 40 nm); Coral (ex: 550 nm, em: 595 \pm 40 nm). (b). Signal to background noise ratio of
776 the first acquired image depicting the brightness of the system and the quality of obtain images. Signal
777 to background noise ratios were calculated from fluorescence intensity values extracted from images
778 using same region of interest from n = 3 independent injections. The value of each measurement is
779 shown as a colored dot. The error bars correspond to \pm 1 standard deviation. (c) Fluorescence intensity
780 decay curves over the time. Data represent average values \pm 1 standard deviation extracted from images
781 from n = 3 independent experiments. (d) Representative micrographs taken during the experiment.
782 Results were found similar in n = 3 independent experiments. Scale bar is 30 μ m.