



HAL
open science

Quinolimide-based peptide biosensor for probing p25 in vitro and in living cells

Francisco Fueyo-González, Rosario Herranz, Simona Plesselova, Maria Giron, Rafael Salto, Jose Manuel Paredes, Angel Orte, May C Morris, Juan González-Vera

► To cite this version:

Francisco Fueyo-González, Rosario Herranz, Simona Plesselova, Maria Giron, Rafael Salto, et al.. Quinolimide-based peptide biosensor for probing p25 in vitro and in living cells. *Sensors and Actuators B: Chemical*, 2021, 339, pp.129929. 10.1016/j.snb.2021.129929 . hal-04570407

HAL Id: hal-04570407

<https://hal.science/hal-04570407v1>

Submitted on 22 Jul 2024

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

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



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 **Quinolimide-based peptide biosensor for probing p25 *in vitro* and in living** 2 **cells**

3 Francisco Fueyo-González^{a,e}, Rosario Herranz^a, Simona Plesselova^b, Maria D. Giron^b, Rafael
4 Salto^b, Jose Manuel Paredes^c, Angel Orte^c, May C. Morris^{d,*}, and Juan A. González-Vera^{a,c,d,*}

5 ^a*Instituto de Química Médica (CSIC). Juan de la Cierva 3, 28006 Madrid, Spain.*

6 ^b*Departamento de Bioquímica y Biología Molecular II, Facultad de Farmacia, Universidad de Granada.*
7 *Campus Cartuja, 18071, Granada, Spain*

8 ^c*Departamento de Fisicoquímica, Unidad de Excelencia de Química Aplicada a Biomedicina y Medioambiente,*
9 *Facultad de Farmacia, Universidad de Granada, Campus Cartuja, 18071, Granada, Spain.*

10 ^d*Institut des Biomolécules Max Mousseron, IBMM-UMR 5247, Université de Montpellier, Faculté de*
11 *Pharmacie, 15 Av. Charles Flahault, 34093 Montpellier, France.*

12 **Corresponding authors E-mail addresses: gonzalezvera@ugr.es (J. A. González-Vera);*
13 *may.morris@umontpellier.fr (M. Morris)*

14

15 **ABSTRACT**

16 CDK5 kinase is activated through interactions with different partners including the p35/p25 regulators.
17 Active CDK5 plays a key role in several neuronal functions and its hyperactivity contributes to a
18 variety of neurodegenerative processes and several human cancers, in particular glioblastomas and
19 neuroblastomas. In order to probe partners that interact with CDK5, we designed and synthesized
20 fluorescent quinolimide-labelled peptides derived from the C-helix of CDK5, which were
21 implemented to probe CDK5 partners *in vitro* and by *in cellulo* imaging in U87 glioblastoma and N2a

^eCurrent address: Department of Medicine, Translational Transplant Research Center, Immunology Institute, Icahn School of Medicine at Mount Sinai, New York, USA.

22 neuroblastoma cells. Ectopic expression of a NIR fluorescent protein miRFP670 fusion with p25 (p25-
23 miRFP670) in N2a cells induced FRET between a quinolimide-labelled peptide biosensor and p25-
24 miRFP670, and fluorescence intensity was proportional to the expression level of p25, thereby
25 demonstrating the potential of the quinolimide-labelled peptide biosensor to report on p25 relative
26 abundance.

27 *Keywords:* Fluorescence probes; Quinolimide derivatives; CDK5; p25 sensors; FRET imaging;
28 Protein-protein interactions.

29

30 **1. Introduction**

31 CDK5 is a serine/threonine kinase, member of the cyclin-dependent kinase (CDK) family, which
32 is expressed ubiquitously, but primarily in post-mitotic neurons, where it is activated by the
33 neurospecific activators p35/p25 and p39/p29 [1]. CDK5 has also been reported to interact with
34 several cyclin partners, including cyclins E, I and Y [2]. CDK5 is mostly present in the central nervous
35 system, where it induces the extension of neurites, neuronal maturation, synapse formation in brain
36 development, synaptic plasticity, axonal guidance, neuronal development and differentiation, and is
37 involved in autophagy regulation [3, 4]. CDK5 activation occurs after the proteolytic cleavage of p35
38 or p39 protein partners to p25 or p29, by calpain and stimulated by Ca^{2+} , which is released into the
39 cytoplasm [5, 6]. p25 does not present sequence similarity with cyclins, but adopts a similar fold and
40 binds to CDK5 at the same interface as other CDKs bind cyclins, known as the C-helix.

41 CDK5 hyperactivity is related with the onset and development of several neurodegenerative
42 diseases, inducing neuronal death [3, 7-9]. Diverse studies have demonstrated the involvement of the
43 CDK5 hyperactivation by p25 in Alzheimer's disease, Parkinson's disease and amyotrophic lateral
44 sclerosis (ALS). In fact, CDK5 participates in phosphorylation of tau protein [9-11]. It also contributes
45 to hyperphosphorylation of α -synuclein and parkin, and to the consequent generation of Lewy bodies
46 in Parkinson's disease [12, 13], and further induces neuronal loss in ALS [14]. Recently, the

47 involvement of CDK5 in neuronal death during cerebral ischemia has also been described [15].
48 Furthermore, numerous studies report upregulation or hyperactivation of CDK5 in several types of
49 human cancers, highlighting the importance of this kinase as a drug target for cancer therapy [3, 9, 16-
50 18]. Abnormal expression or hyperactivation of CDK5 is associated with tumorigenesis and tumor
51 progression, including cancer stem cells, by promoting proliferation, migration and angiogenesis.
52 CDK5 activity participates in control of the DNA damage pathway, resistance to cancer chemotherapy
53 and anti-tumor immunity [16, 17]. In particular, recent studies suggest that the expression and
54 hyperactivity of this kinase are involved in the development and degree of aggressiveness of
55 glioblastomas and neuroblastomas [19, 20]. CDK5 is an invaluable tumorigenesis and glioma
56 progression biomarker [20]. Therefore, CDK5 constitutes an attractive pharmacological target in
57 neurodegenerative disorders (Parkinson's, Alzheimer's, Huntington's and ALS) and oncology. Hence
58 development of a fluorescent biosensor of CDK5 partners involved in its activation would constitute
59 an attractive means of reporting on aberrant or excessive interactions of CDK5 with its partners and its
60 potential dysregulation in pathological conditions.

61 Environment-sensitive fluorophores and in particular solvatochromic dyes have been commonly
62 employed in the development of biosensors for probing protein-protein interactions [21, 22].
63 Photophysical properties (e.g. emission wavelengths, quantum yields and fluorescence lifetimes) of
64 these dyes are highly dependent on the polarity of their neighboring environment, exhibiting, in
65 general, very low fluorescence emission in water and polar solvents, in contrast to that in non-polar
66 environments. This high sensitivity to their surrounding microenvironment makes them suitable for
67 reporting on molecular interactions with high signal-to-noise ratio. Since the interfaces of protein-
68 protein interactions are usually hydrophobic, solvatochromic fluorophores sense these interactions
69 through significant increases in fluorescence, relative to their free exposure to the solvent.

70 In this field, we have described a new family of highly solvatochromic and fluorogenic
71 fluorophores based on a 9-methoxy-quinolimine scaffold (Figure 1, **A**), which demonstrated
72 sensitivity for probing partners of CDK5 in glioblastoma cells [23]. The photophysical properties of

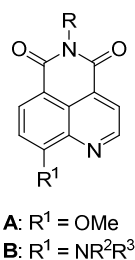
73 these fluorophores have been improved by replacing the electron donor 9-methoxy group by diverse
74 amino groups (Figure 1, **B**). Among the studied amino groups, quinolimides with a methylamino or
75 the cyclic amine azetidine showed the best photophysical properties. Specifically, 9-azetidiny-5-
76 butyl-quinolimide (Figure 1, **B**: R = *n*Bu, R¹ = azetidine) has demonstrated high potential for *in vivo*
77 sensing β -amyloid aggregation in a zebrafish model, enabling to identify the appearance of different
78 types of early pre-amyloid oligomers [24]. In view of the solvatochromic and fluorogenic behavior of
79 9-methylamino and 9-azetidiny-quinolimides, we decided to prepare bromoacetamide derivatives of
80 these fluorophores as peptide labelling reagents and to implement their use to develop a CDK5-based
81 peptide biosensor of p25 level by FRET imaging in cultured cells.

82

83

84

85



86

87

Figure 1. General structure of quinolimide-based fluorophores.

88

89 2. Experimental Section

90 2.1. Synthetic Materials and Methods

91 Complete information on the synthesis, photophysical and NMR characterization data of quinolimides
92 **2–5** (see Scheme 1) has been included in the Supplementary Material, as well as peptide labelling and
93 titration experiments.

94 2.2. Colocalization of quinolimide labelled biosensor (Pep1-K5-5) with endogenous p25 in U87 95 glioblastoma cells

96 A previously reported protocol was followed [23]. Pep1-CDK5 peptide labelled with **5** (Pep1-K5-
97 **5**, 2 μ M) was added to U87 cells. Indirect immunofluorescence was carried out with rabbit polyclonal

98 anti-p25 using Alexa Fluor 647-conjugated secondary antibodies and nuclei were stained with
99 Hoechst. Cells were visualized using a Zeiss microscope with a CoolSnap camera and images were
100 obtained with MetaMorph software.

101 *2.3. Generation of a eukaryotic expression vector for p25 fused to the miRFP660 fluorescent*
102 *protein (pmiRFP670-N-P25)*

103 p25 coding sequence was amplified by PCR from plasmid GST-p25 (a gift from Harish Pant,
104 Addgene plasmid # 24896) that encodes for a truncated version of p35 protein [25]. p25 sequence,
105 without the stop codon was amplified from GST-p25 by PCR using *Pfu* polymerase and primers
106 including a 5' *Bam*HI and 3' *Eco*RI restriction sites. In addition, the forward primer included a Kozak
107 sequence plus an initiation codon (Forward primer: 5'-ggatcccaccatgGCCAGCCCCCGCCG-3' and
108 Reverse primer: 5'-GaattcgCCGATCCAGGCCTAGGAG-3' where the lowercase letters correspond
109 to the restriction sites, kozak sequence and initiation codon added). The PCR fragment was subcloned
110 using *Bam*HI and 3' *Eco*RI restriction sites into the *Bgl*II and *Eco*RI restriction sites in the pmiRFP670-
111 N1 vector (a gift from Vladislav Verkhusha, Addgene plasmid # 79987), a eukaryotic expression
112 vector that encodes the monomeric near-infrared fluorescent protein miRFP670 [26]. The resulting
113 plasmid was termed pmiRFP670-N-p25 and its sequence was confirmed by automatic sequencing.

114 *2.4. In cellulo probing expression levels of p25 in N2a cells using dual-color ratiometric and*
115 *fluorescence lifetime (FLIM) imaging*

116 Murine neuroblastoma Neuro-2a cells (N2a; ATCC No. CCL-131) were cultured in Dulbecco's
117 Modified Eagle's medium (DMEM) supplemented with glutamine plus (2 mM), FBS (10 %),
118 penicillin (100 units/mL) and streptomycin (0.1 mg/mL) in a CO₂ atmosphere (5 %). Prior to
119 transfection either with plasmid pmiRFP670-N-P25 or pmiRFP670-N1, cells were seeded onto
120 coverslips in 6 well plates at a density of 2.3×10^5 cells/well for 24 h to obtain an 80–90 % cell
121 confluence. For transfection experiments, pmiRFP670-N1 and pmiRFP670-N-P25 plasmids (4.3
122 µg/well) were mixed with LP2000 (10 µL) for 30 min at room temperature (100 µL of total volume).

123 Next, the mixture was diluted with DMEM (without FBS) to 1 mL and added to the wells. Cells were
124 added the polyplexes and incubated for 5 h. Afterwards medium was removed and cells were grown in
125 DMEM plus FBS (10 %) for 24 h. Transfected cells were used for fluorescence microscopy and image
126 analysis. For differentiation experiments, medium was replaced with DMEM supplemented with 1%
127 FBS for 24h. For the experiments using rosiglitazone, N2a cells were treated with rosiglitazone 200
128 μ M for 24h.

129 2.5. *Dual-color FLIM studies*

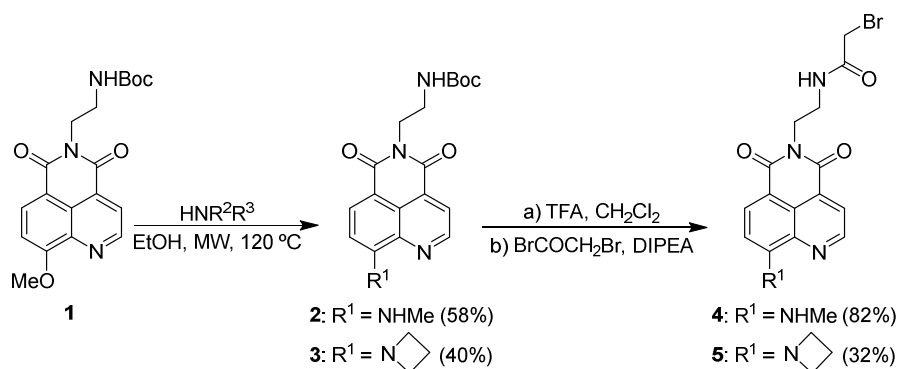
130 Dual-color FLIM was performed on a MicroTime 200 system (PicoQuant GmbH) as previously
131 described [24]. Two pulsed interleaved excitation lasers at $\lambda_{\text{ex}}= 470$ nm and $\lambda_{\text{ex}}= 635$ nm were
132 employed and fluorescence was split into two detection channels with a 600DCXR dichroic mirror.
133 The green channel was defined with a 550/40 bandpass filter whereas a 685/70 bandpass filter was
134 used for red channel. FLIM imaging analyses were carried out employing SymphoTime 32 (PicoQuant
135 GmbH) and home-coded scripts in FiJi (distribution of ImageJ [27]).

136

137 3. Results and discussion

138 3.1. *Synthesis of peptide labelling reagents*

139 As shown in Scheme 1, the 9-amino quinolimides **2** and **3** were obtained by replacement of the 9-
140 methoxy group of quinolimide **1** [23] by the corresponding amine, via aromatic nucleophilic
141 substitution [28], under microwave activated heating at 120 °C. After removal of the protecting Boc
142 group from **2** and **3**, by treatment with TFA in CH_2Cl_2 , the reaction at room temperature with
143 bromoacetic acid bromide in the presence of DIPEA, as acceptor of the generated BrH, led to the
144 corresponding bromoacetamides **4** and **5** with 82 and 32 % yield, respectively.



145

146 **Scheme 1.** Synthesis of the thiol-reactive bromoacetamides **4** and **5**.

147

148 **3.2. Photophysical properties of bromoacetamides 4 and 5**

149 UV absorption and fluorescence emission properties of bromoacetamide derivatives **4** and **5** were
 150 determined in dioxane, as nonpolar solvent, and water and are shown in Table 1. As expected the
 151 fluorescence emission of **4** and **5** was significantly higher in dioxane than in water, as well as their
 152 respective lifetimes. Moreover, these dyes display very large Stokes shifts (> 100 nm).

153 **Table 1.** Photophysical properties of the bromoacetamides **4** and **5**

Compd ^a	Solvent	$\lambda_{\text{max}}^{\text{abs}}$ (nm)	ϵ (M ⁻¹ cm ⁻¹)	$\lambda_{\text{max}}^{\text{em}}$ (nm)	$\Phi_{\text{F}}^{\text{b}}$	τ (ns) ^c
4	Dioxane	463	15300 ± 1000	583	0.029 ± 0.002	1.80 ± 0.01
	H ₂ O	483	11100 ± 700	617	<0.001	0.20 ± 0.02
5	Dioxane	474	14200 ± 900	583	0.120 ± 0.008	3.96 ± 0.08
	H ₂ O	492	11200 ± 700	623	<0.001	0.68 ± 0.06

154 ^aMeasured in duplicate at a 12 μM concentration. ^bQuantum yields calculated with reference to coumarin 153 (in EtOH).
 155 ^cFluorescence lifetimes obtained with $\lambda_{\text{ex}} = 485$ nm, as global fits of three fluorescence decay traces collected at $\lambda_{\text{max}}^{\text{em}} - 10$
 156 nm, $\lambda_{\text{max}}^{\text{em}}$, and $\lambda_{\text{max}}^{\text{em}} + 10$ nm. The reported values are the mean of the intensity-weighted average fluorescence lifetimes at
 157 the three different wavelengths from triexponential decay fittings. The associated error is the standard deviation of the mean.

158

159

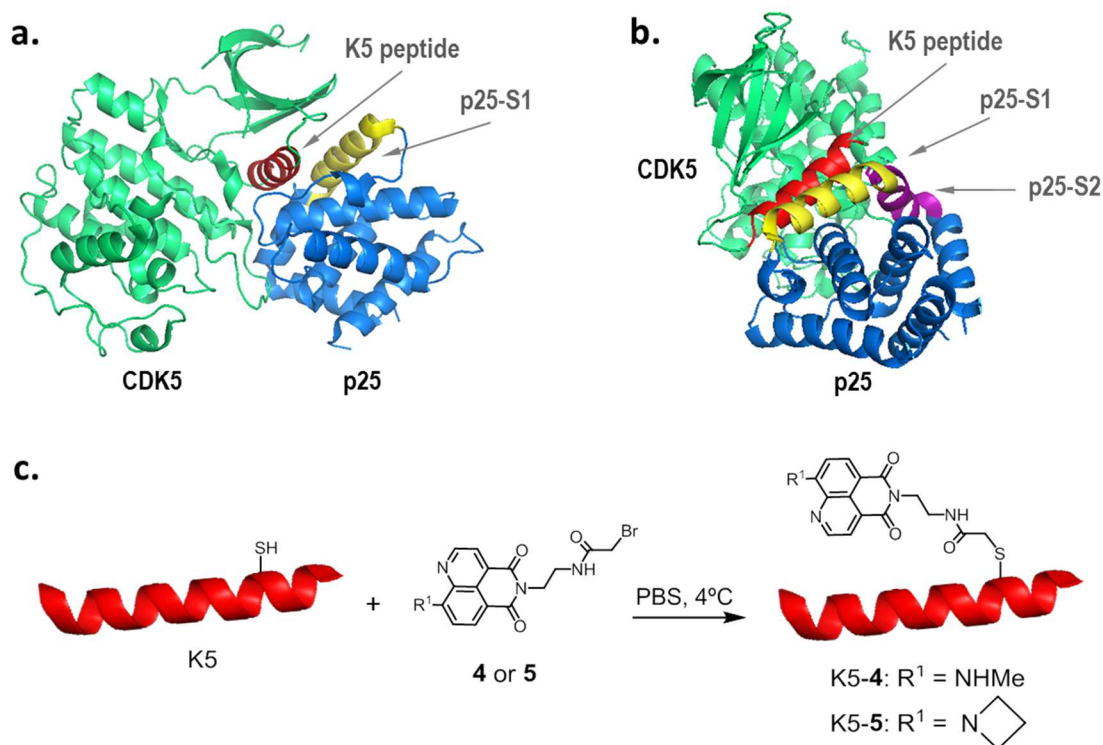
160

161

162

163 3.3. Fluorescent peptide design, labelling and in vitro study of the CDK5/p25 interaction

164 As shown in Figure 3, the crystal structure of the CDK5/p25 complex shows that CDK5 binds p25
165 through the C-helix (PISTVRE, in red) of CDK5 and the α helix of p25 (in yellow) [29, 30].
166 Therefore, to study the interaction between the C-helix of CDK5 and its partners, we labelled a peptide
167 derived from the C-helix of CDK5 (K5 peptide, GVPSSALREICLLK [23]), which bears a single
168 cysteine residue, with the prepared bromoacetamides **4** and **5**, respectively (Figure 3c). The resulting
169 labelled peptides K5-**4** and K5-**5** were then titrated with a peptide derived from the interacting α helix
170 of p25 (p25-S1 peptide, KEAFWDRCLSVINLM [23]). In the absence of this peptide, the emission of
171 peptides K5-**4** (Figure 4a) and K5-**5** (Figure 4b) was very low; however, it increased significantly in
172 the presence of increasing concentrations of the p25-S1 peptide. Addition of p25-S1 peptide to
173 labelled peptides K5-**4** and K5-**5** produced hypsochromic shifts of 38 and 37 nm (from red to orange)
174 and 21.6- and 20.2-fold fluorescence emission intensity enhancement, respectively, indicative of an
175 effective interaction with p25-S1.



176

177 **Figure 3.** (a and b) Cartoon representation of the CDK5/p25 complex (PDB 1UNG), in two different poses, with
178 the interface between CDK5 (green) and p25 (blue) mediated by contacts between the C helix of CDK5
179 (PSTAIRE; red), and the α helix of p25 (yellow)[30]. Color code of peptides derived from CDK5/p25 interaction
180 employed in this work: p25-S1 peptide in yellow, p25-S2 peptide in purple and K5 peptide in red. (c) Scheme of
181 peptide labelling.

182

183 Binding constants, K_d , were obtained through the fluorescence titration curves as described in the
184 Supplementary Material. The K_d value for K5-4 was $420 \pm 80 \mu\text{M}$, while K5-5 exhibited a K_d value of
185 $49 \pm 15 \mu\text{M}$, which, importantly, represents a 8.5-fold increase in affinity compared to that of K5-4
186 and 7-fold increase respect to that of our previously reported 9-methoxy-quinolimide-based biosensor
187 [23]. Moreover, the titration of K5-4 and K5-5 with an unrelated peptide
188 (VESSDTIDNVKSKIQDKEGC [31]) (Figure S3) did not produce a fluorescence increase,
189 demonstrating that the probe is not involved in a false interaction and, therefore, highlighting that
190 binding of the labelled peptides is selective for the p25-S1 peptide. As a negative control, we titrated
191 K5-5 with a peptide derived from another α helix close to the interacting α helix of p25 but not
192 directly involved in the CDK5/p25 interaction (p25-S2 peptide, CSKMLQINAD), which showed a
193 negligible binding (Figure 4c; $K_d > 6000 \mu\text{M}$). Furthermore, the titration of the K5 peptide labelled
194 with a standard fluorophore with low solvatochromic behavior, such as fluorescein (FITC), only led to
195 1.5-fold increase in fluorescence, with no related spectral shift (Figure S3). These results show that
196 both K5-4 and K5-5 peptides constitute useful tools for studying partners that interact with the C-helix
197 of CDK5, in particular the azetidine derivative K5-5, due to its greater brightness, longer excitation
198 and emission wavelengths, larger fluorescence lifetime and greater affinity for the peptide derived
199 from p25-S1.

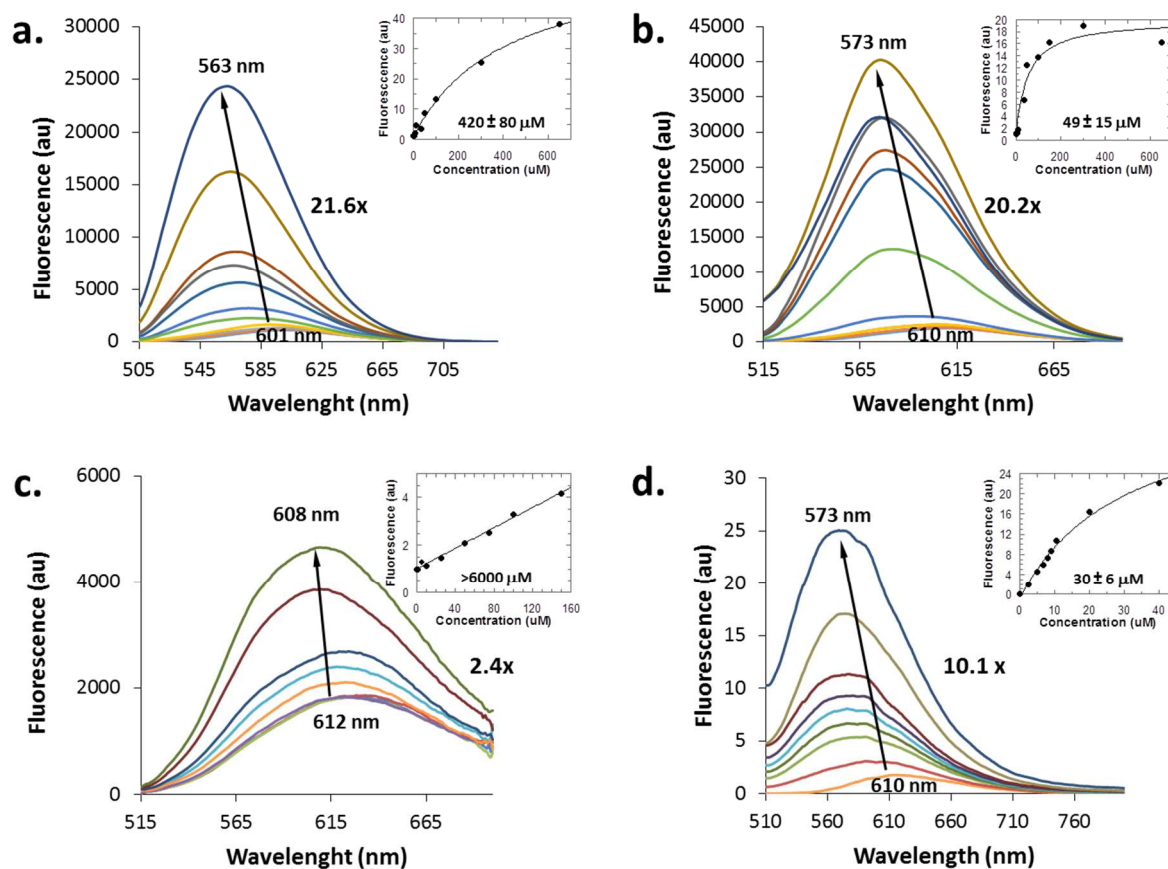
200

201

202

203

204



205

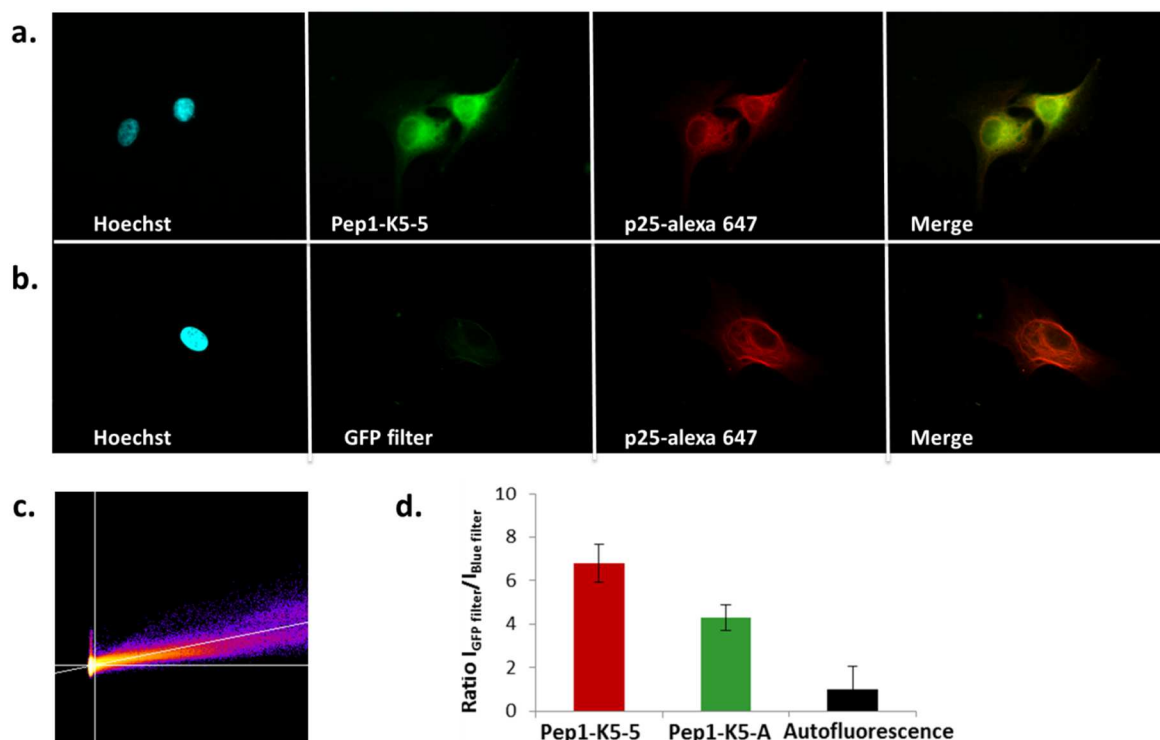
206 **Figure 4.** (a) Titration of K5-4 (5 μM) with the p25-S1 peptide ($\lambda_{\text{ex}}=480\text{ nm}$) and curve fit. (b) Titration of K5-5
 207 (5 μM) with the p25-S1 peptide ($\lambda_{\text{ex}}=490\text{ nm}$) and curve fit. (c) Titration of K5-5 (5 μM) with the p25-S2
 208 peptide ($\lambda_{\text{ex}}=490\text{ nm}$) and curve fit. (d) Titration of Pep1-K5-5 (0.2 μM) with GST-p25 protein ($\lambda_{\text{ex}}=490\text{ nm}$)
 209 and curve fit. Insets a-d: plots of fluorescence intensity versus concentration of the peptide. Lines represent
 210 results from non-linear curve fitting from which the binding constant, K_d , values included in the insets were
 211 obtained.

212

213 3.4. Colocalization of quinolimide labelled biosensor (Pep1-K5-5) with endogenous p25 in U87 214 glioblastoma cells

215 Next, we investigated whether K5 labelled with the 9-azetidiny-quinolimide **5** (K5-5) could be
 216 used as a probe for the *in cellulo* imaging of CDK5 partners in U87 glioblastoma cells. As expected,
 217 the K5-5 peptide, by itself, did not penetrate into cells. Therefore, a fusion of the cell-penetrating
 218 peptide Pep1 [32, 33] with the K5 peptide was synthesized and was then labelled with
 219 bromoacetamide **5**. Prior to the *in cellulo* assay, we studied the *in vitro* interaction between labelled

220 peptide Pep1-K5-5 (KETWWETWWTEKKGVPSSALREIC(5)LLK) and recombinant GST-p25. The
 221 titration of Pep1-K5-5 with GST-p25 led to a hypsochromic shift of 37 nm and 10.1-fold increase in
 222 fluorescence, with a K_d value of $30 \pm 6 \mu\text{M}$, which indicated an effective binding to p25 protein
 223 (Figure 4d). Pep1-K5-5 peptide readily penetrated into living U87 cells, and its colocalization with
 224 endogenous p25 was investigated by fluorescence microscopy following indirect immunofluorescence
 225 with a p25 antibody labelled with the Alexa Fluor 647 fluorophore in U87 cells overlaid with the
 226 Pep1-K5-5 peptide. The results showed a complete overlay of the signal of Pep1-K5-5 observed
 227 through the GFP filter with that of Alexa Fluor 647-labelled secondary/anti-p25 primary antibodies
 228 (Figure 5a). The calculated Pearson colocalization coefficient was 0.93 ± 0.03 (Figure 5c). In contrast,
 229 cells which had not been treated with the Pep1-K5-5 peptide did not reveal any signal through the GFP
 230 channel (Figure 5b). Remarkably, the Pep1-K5-5 peptide led to a 6.3-fold fluorescence increase,
 231 inferring it interacts with p25 *in cellulo*. As shown in Figure 5d, this represents a significant
 232 improvement compared to our previously reported 9-methoxy-quinolimide-based CDK5 probe (Pep1-
 233 K5-A) [23].



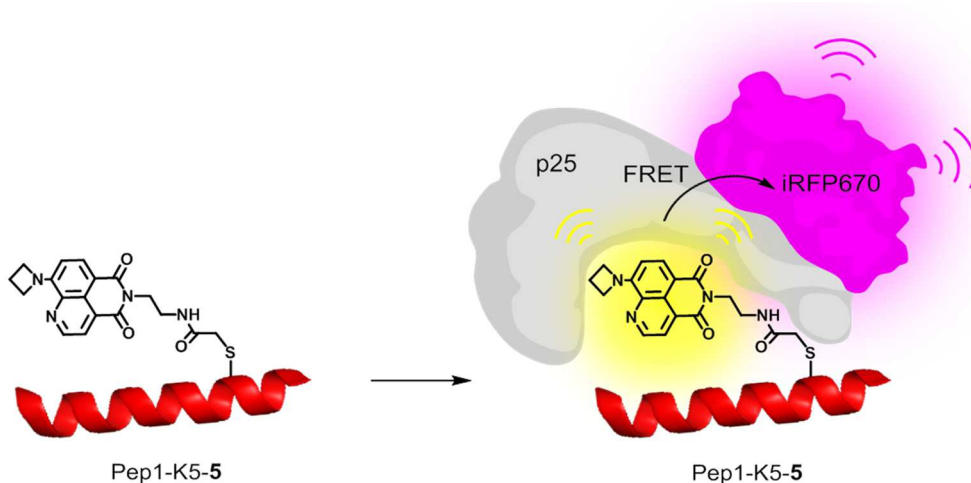
234
235

236 **Figure 5.** Colocalization of Pep1-K5-5 with p25. (a) Indirect immunofluorescence of p25 in cells treated with
237 Pep1-K5-5. (b) Indirect immunofluorescence of p25 in cells. Images in the green channel (GFP filter) appeared
238 with negligible signal. (c) Graph representing the intensity in the two channels used to calculate Pearson
239 colocalization coefficient. (d) Intensity ratio $I_{\text{GFP filter}}/I_{\text{Blue filter}}$ of Pep1-K5-5 and the analogue 9-methoxy-
240 quinolimide labelled Pep1-K5 peptide (Pep1-K5-A) [23].

241

242 3.5. *Reporting different levels of p25 in cellulo via FRET between Pep1-K5-5 and ectopically*
243 *expressed iRFP670-p25 in N2a neuroblastoma cells*

244 Finally, we decided to assess the potential of the 9-azetidiny-quinolimide labelled peptide Pep1-
245 K5-5 to report on different levels and expression patterns of the p25 protein in cultured cells. For this
246 purpose, we engineered a FRET-based system composed by our 9-amino-quinolimide labelled peptide
247 derived from CDK5 (Pep1-K5-5), as FRET donor, and p25 protein fused to the NIR fluorescent fusion
248 protein miRFP670 (p25-miRFP670), as FRET acceptor. miRFP670 was chosen based on its spectral
249 properties [34], which ensure a suitable FRET pair with the 9-azetidiny-quinolimide dye thanks to a
250 good spectral overlap. In this system, when the labelled peptide Pep1-K5-5 would bind p25-
251 miRFP670, we hypothesized the probe would become embedded in a hydrophobic environment which
252 would increase its fluorescence and transfer the energy to miRFP670, thereby resulting in NIR
253 emission (Figure 6), together with a decrease in the fluorescence lifetime of 5. The implementation of
254 the proposed FRET system would formally demonstrate that the CDK5-derived peptide biosensor
255 indeed recognizes and responds to differences in p25 abundance in cultured cells. Moreover it would
256 provide the opportunity to develop an *in cellulo* screen of compounds, which might interfere with the
257 CDK5/p25 interaction, of potential therapeutic utility.



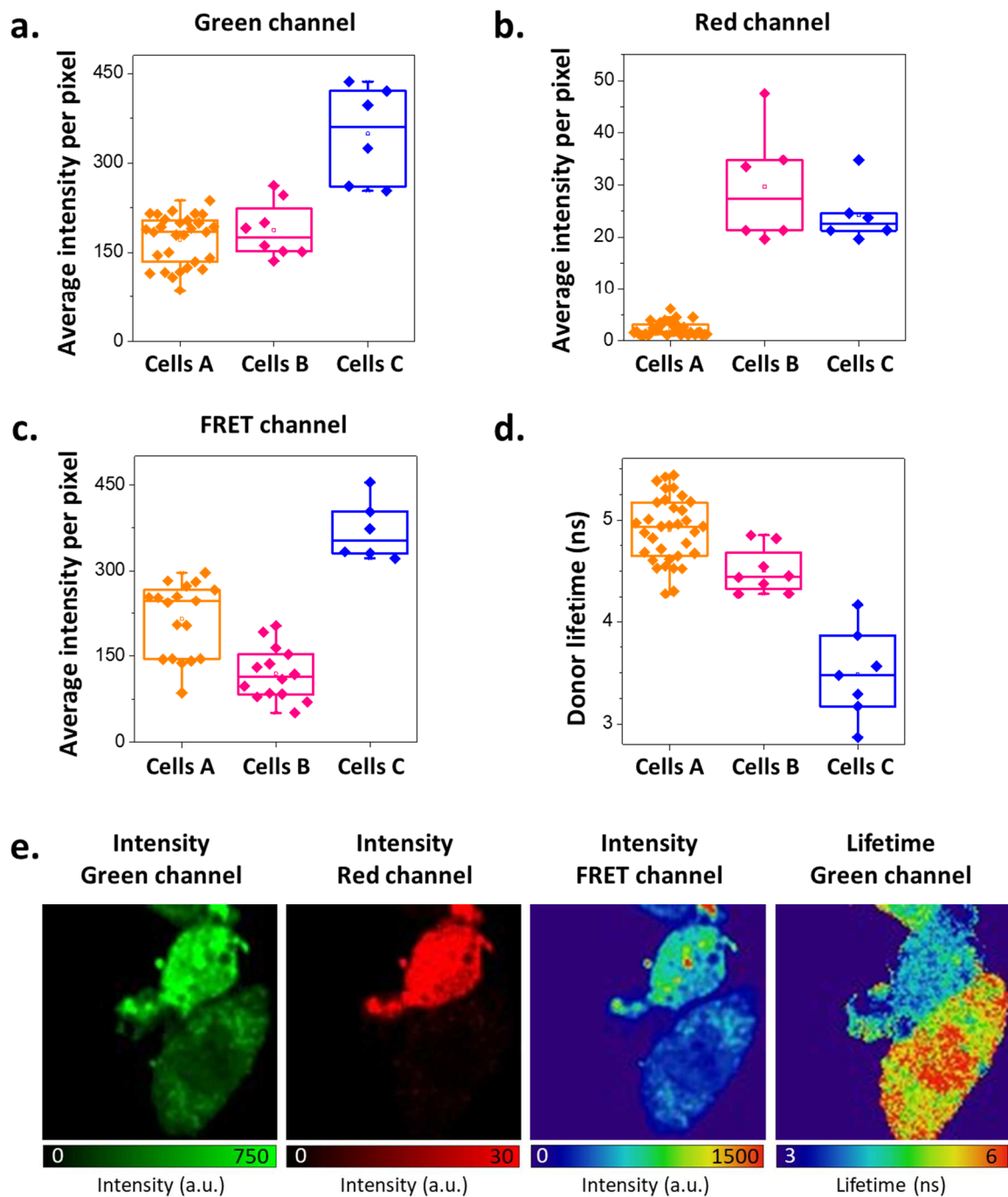
258

259 **Figure 6.** Schematic representation of the FRET-based biosensor for K5/p25 interaction. Upon binding of the
 260 Pep1-K5-5 peptide to the p25-miRFP670 target protein, the donor dye **5** would transfer its excitation energy to
 261 the fluorescent fusion protein, resulting in NIR emission of the acceptor.

262

263 With this experimental design in mind, N2a neuroblastoma cells expressing physiological levels of
 264 p25 [35] were either mock-transfected or transfected with pmiRFP670-N-p25 or pmiRFP670-N1
 265 plasmids. The N2a mock-transfected cells (cells A) as well as the pmiRFP670-N1 transfected cells
 266 (cells B) express basal, physiological levels of p25. On the contrary, the pmiRFP670-N-p25
 267 transfected cells (cells C) overexpress p25 fused to the miRFP670 fluorescent protein. Three different
 268 microscope configurations (Figure 7) were selected in order to separate the emission of the
 269 fluorescently-labelled Pep1-K5-5 peptide ($\lambda_{\text{ex}} = 470 \text{ nm}$, $\lambda_{\text{em}} = 550/40 \text{ nm}$; Green channel, Figure 7a),
 270 the emission of the NIR protein miRFP670 ($\lambda_{\text{ex}} = 635 \text{ nm}$, $\lambda_{\text{em}} = 685/70 \text{ nm}$; Red channel, Figure 7b)
 271 and the last channel corresponding to the emission due to FRET ($\lambda_{\text{ex}} = 470 \text{ nm}$, $\lambda_{\text{em}} = 685/70 \text{ nm}$;
 272 FRET channel, Figure 7c). Importantly, we also followed the FRET process by focusing on the
 273 decrease in the donor's fluorescence lifetime by using fluorescence lifetime imaging (FLIM, Figure
 274 7d).

275



276

277 **Figure 7.** Intracellular FRET-based biosensor measurements and images. Box and whisker plots representing the
 278 average fluorescence intensity (a) in the green channel, (b) in the red channel, (c) in the FRET channel and (d)
 279 the fluorescence lifetime of the donor dye **5** in pixels from FLIM images containing Pep1-K5-**5**-labelled peptide
 280 in N2a cells. Boxes represent the 25 and 75%, with a horizontal bar showing the average value. Whiskers
 281 correspond to the minimum and maximum values. (e) Representative dual-color FLIM images containing two
 282 different types of cells, cell C (top) and cell A (below), according to p25 expression levels. The three images on
 283 the left represent the fluorescence intensities from the green, red and FRET channels. The right image represents
 284 the fluorescence lifetime of Pep1-K5-**5**.

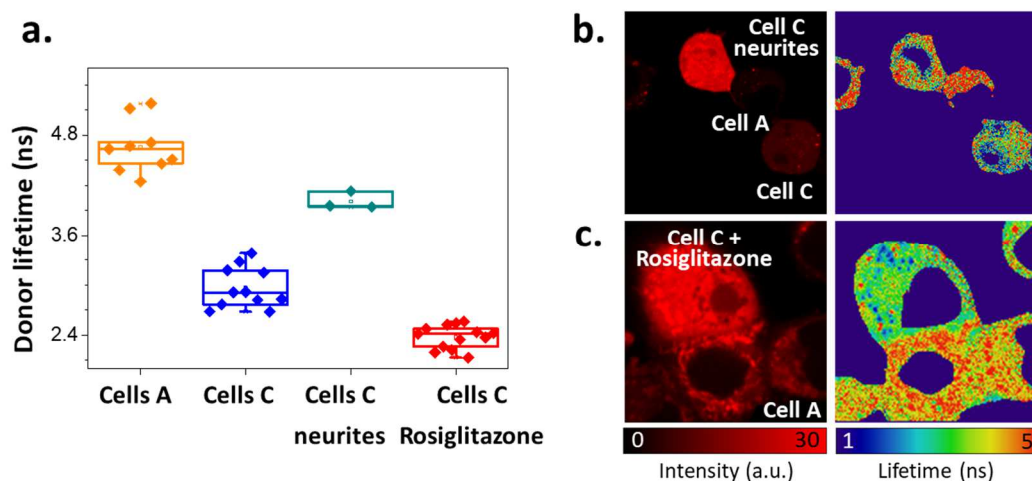
285 As shown in Figure 7a, compared to cells expressing physiological levels of p25 (cells A and B),
286 the binding of Pep1-K5-5 to cells overexpressing p25 (cells C) resulted in a >2.5-fold increase in the
287 emission intensity of the peptide biosensor in the green channel. This increase was associated with the
288 higher concentration of p25 in cells overexpressing this protein, which upon interaction with Pep1-K5-
289 5 promoted an increase in the hydrophobicity of the local environment of the fluorescent probe and,
290 therefore, increases its fluorescence emission. Importantly, this fluorescence increase successfully
291 allowed us to distinguish between cells with different intracellular concentrations of p25. Furthermore,
292 as expected, when the red channel fluorescence was studied (Figure 7b), low fluorescence levels were
293 detected in non-transfected N2a cells (cells A), while similar levels of NIR fluorescence were detected
294 on the cells transfected either with the pmiRFP670-N1 (cells B) or pmiRFP670-N-P25 plasmids (cells
295 C). As a consequence of the energy transfer from the 9-amino-quinolimide labelled peptide Pep1-K5-5
296 to p25-miRFP670, the intensity in the FRET channel, using compound 5 excitation wavelength but
297 selecting the far-red emission channel (Figure 7c), was higher in the population of “C” cells than in the
298 population of “A” cells, where FRET was not possible due to the absence of the FRET acceptor p25-
299 miRFP670. Furthermore, when FRET was analyzed in the pmiRFP670-N1 transfected cells (“B”
300 cells), the signal was absent since there was no miRFP670 protein fused to the p25 moiety available
301 for energy transfer from the 9-amino-quinolimide labelled peptide Pep1-K5-5 onto miRFP670.

302 The interaction of Pep1-K5-5 with the p25-miRFP670 protein not only produced an increase in
303 FRET fluorescence intensity, but more significantly produced a decrease on the fluorescence lifetime
304 of Pep1-K5-5, which is an unequivocal feature of actual FRET, and is evidenced by FLIM
305 microscopy. Figure 7d shows the fluorescence lifetime obtained from the green channel for cell
306 populations A, B and C. The fluorescence lifetime of the donor quinolimide in the Pep1-K5-5
307 biosensor approximately decreased from 4.9 or 4.5 in cells expressing physiological levels of p25,
308 cells A and B respectively, to 3.7 ns in cells C, which represents an average FRET efficiency of 0.24
309 (estimated with respect to the lifetime of Pep1-K5-5 in cells A). These results confirmed the presence
310 of FRET between Pep1-K5-5 and p25-miRFP670 in cells overexpressing p25, and, therefore,

311 demonstrated the intracellular binding between the CDK5-based peptide Pep1-K5-5 and its regulatory
312 partner p25. The significant decrease in the fluorescence lifetime of the peptide biosensor confirms the
313 utility of our FRET based system to probe the CDK5/p25 interaction and to detect ectopic
314 overexpression of p25. As expected, in the population of “B” cells, where only miRFP670 was
315 expressed, no lifetime changes in the donor due to FRET were observed. Figure 7e shows a
316 representative example of a dual color FLIM experiment in which two different types of cells were
317 detected. In this example a cell is characterized by a high intensity in the red and the FRET channels
318 and a low donor lifetime (cell C), whereas the other cell shows negligible intensity in the red and the
319 FRET channel and a high donor lifetime (cell A). This indicates different p25 levels in these two cell
320 populations.

321 To demonstrate the usefulness of the measurement system based on the interaction of Pep1-K5-5
322 with the p25-miRFP670 in physio-pathological conditions, N2a cells transfected with the pmiRFP670-
323 N-P25 plasmid (cells C) were either differentiated to neurites or treated with rosiglitazone, and then
324 the donor lifetime of the sensor was compared to that found in the N2a untransfected cells (cells A)
325 (Figure 8). The differentiation of N2a cells to neurites involves an increase in the endogenous levels of
326 p25 that translated into an enhanced CDK5 activity [36]. Therefore, an increase in the lifetime could
327 be expected due to competition between the increased levels in p25 in the differentiated neurites and
328 the p25-miRFP670 protein for binding to the Pep1-K5-5 probe. On the contrary, it has been described
329 that in neuroblastoma cells, incubation with thiazolidinediones such as rosiglitazone [37] increases the
330 degradation rate of p25 decreasing its intracellular levels. Following the same rationale, this should
331 translate in a decrease in the fluorescence lifetime of C cells treated with rosiglitazone. Our results
332 confirmed that the sensor can be used to follow expression levels of p25 in physiological conditions
333 (differentiation to neurites) or after pharmacological treatment (incubation with rosiglitazone),
334 validating our measurement system in cells cultures in response to intracellular changes of p25.

335



336

337 **Figure 8.** Intracellular FRET-based biosensor measurements in physio-pathological conditions. (a) Box and
 338 whisker plots representing the fluorescence lifetime of the donor dye **5** in pixels from FLIM images containing
 339 Pep1-K5-**5**-labelled peptide in N2a cells. Boxes represent the 25 and 75%, with a horizontal bar showing the
 340 average value. Whiskers correspond to the minimum and maximum values. N2a cells were transfected with the
 341 pmiRFP670-N-P25 plasmid (cells C) and were either differentiated to neurites (Cells C neurites) or treated with
 342 rosiglitazone (Cells C rosiglitazone). Cells A were untransfected N2a cells. (b and c) Representative dual-color
 343 FLIM images (left column: fluorescence intensity from the red channel; right column: fluorescence lifetime of
 344 Pep1-K5-**5**) containing (b) three different types of cells, cell C differentiated to neurites (top), cell A (middle)
 345 and cell C undifferentiated (below), or (c) two different types of cells, cell C treated with rosiglitazone (top) and
 346 cell A (below), according to p25 expression levels.

347

348 4. Conclusions

349 The results herein described illustrate the remarkable potential of highly solvatochromic and
 350 fluorogenic 9-(substituted amino)-quinolimide-based fluorophores, and particularly 9-azetidiny-
 351 quinolimides, as tools for fluorescence peptide labelling, and to obtain biosensors of cell cycle
 352 regulators. Specifically we describe the design and synthesis of CDK5-based peptide biosensors
 353 labelled with 9-methylamino- or 9-azetidiny-quinolimides, which showed *in vitro* μ M affinity for
 354 p25, the regulatory partner of CDK5. We have further shown that the 9-azetidiny-quinolimide
 355 labelled biosensor (Pep1-K5-**5**) colocalized with p25 in U87 glioblastoma cells. Finally we have
 356 developed a FRET strategy combining our peptide biosensor and ectopic expression of the NIR
 357 fluorescent miRFP670-p25 fusion protein, which has enabled to formally demonstrate the interaction
 358 between the Pep1-K5-**5** biosensor and ectopically expressed miRFP670-p25 in neuroblastoma N2a

359 cells. Our studies have shown that the FRET response is correlated with p25 levels. Moreover, the
360 implementation of this system opens the door to its use for the *in cellulo* screening of libraries of
361 compounds for identification of CDK5/p25 modulators of therapeutic interest.

362

363 **Conflict of interest**

364 The authors have no conflicts of interest to declare.

365

366 **Acknowledgements**

367 This work was supported by the Ministerio de Ciencia e Innovación/Agencia Estatal de
368 Investigación grants FU2015-67284-R and PID2019-104366RB-C22, the Ministerio de Ciencia e
369 Innovación/Agencia Estatal de Investigación/European Regional Development Fund grant CTQ2017-
370 85658-R, and the CSIC grant 201580E073, and the CNRS (Centre National de la Recherche
371 Scientifique). We acknowledge the MRI imaging facility (Montpellier, France), a member of the
372 national infrastructure France-BioImaging.

373

374 **Supplementary Material**

375 Supplementary material to this article can be found online at

376

377 **References**

- 378 [1] L.-H. Tsai, I. Delalle, V.S. Caviness, Jr., T. Chae, E. Harlow, P35 is a neural-specific regulatory
379 subunit of cyclin-dependent kinase 5, *Nature*, 371(1994) 419-23.
380 [2] R. Roufayel, N. Murshid, CDK5: Key Regulator of Apoptosis and Cell Survival, *Biomedicines*,
381 7(2019).

382 [3] M. Peyressatre, C. Prevel, M. Pellerano, M.C. Morris, Targeting cyclin-dependent kinases in
383 human cancers: from small molecules to Peptide inhibitors, *Cancers*, 7(2015) 179-237.

384 [4] R. Dhavan, L.H. Tsai, A decade of CDK5, *Nature reviews Molecular cell biology*, 2(2001) 749-59.

385 [5] G. Kusakawa, T. Saito, R. Onuki, K. Ishiguro, T. Kishimoto, S. Hisanaga, Calpain-dependent
386 proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25, *J Biol Chem*, 275(2000)
387 17166-72.

388 [6] M.-s. Lee, Y.T. Kwon, M. Li, J. Peng, R.M. Friedlander, L.-H. Tsai, Neurotoxicity induces cleavage of
389 p35 to p25 by calpain, *Nature* 405(2000) 360-4.

390 [7] S.B. Shelton, G.V.W. Johnson, Cyclin-dependent kinase-5 in neurodegeneration, *J Neurochem*,
391 88(2004) 1313-26.

392 [8] A. Camins, E. Verdaguer, J. Folch, A.M. Canudas, M. Pallas, The role of CDK5/P25
393 formation/inhibition in neurodegeneration, *Drug News Perspect*, 19(2006) 453-60.

394 [9] Z.H. Cheung, N.Y. Ip, Cdk5: a multifaceted kinase in neurodegenerative diseases, *Trends Cell Biol*,
395 22(2012) 169-75.

396 [10] L.-F. Lau, M.K. Ahlijanian, Role of cdk5 in the pathogenesis of Alzheimer's disease, *Neurosignals*,
397 12(2003) 209-14.

398 [11] J. Kanungo, Special Issue on "Cdk5 and Brain Disorders": Prologue, *Brain Disord Ther, Suppl*
399 1(2012) 1-5.

400 [12] J.P. Brion, A.M. Couck, Cortical and brainstem-type Lewy bodies are immunoreactive for the
401 cyclin-dependent kinase 5, *Am J Pathol*, 147(1995) 1465-76.

402 [13] E. Rubio de la Torre, B. Luzon-Toro, I. Forte-Lago, A. Minguez-Castellanos, I. Ferrer, S. Hilfiker,
403 Combined kinase inhibition modulates parkin inactivation, *Hum Mol Genet*, 18(2009) 809-23.

404 [14] M.D. Nguyen, J.-P. Julien, Cyclin-Dependent Kinase 5 in amyotrophic lateral sclerosis,
405 *Neurosignals*, 12(2003) 215-20.

406 [15] D.A. Meyer, M.I. Torres-Altora, Z. Tan, A. Tozzi, M. Di Filippo, V. DiNapoli, et al., Ischemic stroke
407 injury is mediated by aberrant Cdk5, *J Neurosci*, 34(2014) 8259-67.

408 [16] K. Pozo, J.A. Bibb, The Emerging Role of Cdk5 in Cancer, *Trends Cancer*, 2(2016) 606-18.

409 [17] A. Shupp, M.C. Casimiro, R.G. Pestell, Biological functions of CDK5 and potential CDK5 targeted
410 clinical treatments, *Oncotarget*, 8(2017) 17373-82.

411 [18] A. Catania, S. Urban, E. Yan, C. Hao, G. Barron, J. Allalunis-Turner, Expression and localization of
412 cyclin-dependent kinase 5 in apoptotic human glioma cells, *Neuro-oncology*, 3(2001) 89-98.

413 [19] R. Liu, B. Tian, M. Gearing, S. Hunter, K. Ye, Z. Mao, Cdk5-mediated regulation of the PI3K-Akt
414 pathway and glioblastoma cell invasion, *Proc Natl Acad Sci U S A*, 105(2008) 7570-5.

415 [20] R. Yushan, G. Roodrajeetsing, C. Wenjie, D. Yiwu, Z. Tengfei, W.M. Madushi, et al., Insights into
416 the clinical value of cyclin-dependent kinase 5 in glioma: a retrospective study, *World J Surg Oncol*,
417 13(2015) 223.

418 [21] G.S. Loving, M. Sainlos, B. Imperiali, Monitoring protein interactions and dynamics with
419 solvatochromic fluorophores, *Trends Biotechnol*, 28(2010) 73-83.

420 [22] A.S. Klymchenko, Solvatochromic and Fluorogenic Dyes as Environment-Sensitive Probes: Design
421 and Biological Applications, *Accounts of chemical research*, 50(2017) 366-75.

422 [23] J.A. González-Vera, F. Fueyo-González, I. Alkorta, M. Peyressatre, M.C. Morris, R. Herranz, Highly
423 solvatochromic and tunable fluorophores based on a 4,5-quinolimine scaffold: novel CDK5 probes,
424 *Chem Commun* 52(2016) 9652-5.

425 [24] F. Fueyo-González, J.A. González-Vera, I. Alkorta, L. Infantes, M.L. Jimeno, P. Aranda, et al.,
426 Environment-Sensitive Probes for Illuminating Amyloid Aggregation In Vitro and in Zebrafish, *ACS*
427 *Sens*, 5(2020) 2792-9.

428 [25] N.D. Amin, W. Albers, H.C. Pant, Cyclin-dependent kinase 5 (cdk5) activation requires interaction
429 with three domains of p35, *Journal of neuroscience research*, 67(2002) 354-62.

430 [26] D.M. Shcherbakova, M. Baloban, A.V. Emelyanov, M. Brenowitz, P. Guo, V.V. Verkhusha, Bright
431 monomeric near-infrared fluorescent proteins as tags and biosensors for multiscale imaging, *Nature*
432 *communications*, 7(2016) 12405.

433 [27] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, et al., Fiji: an open-
434 source platform for biological-image analysis, *Nature methods*, 9(2012) 676-82.

435 [28] B. Štefane, F. Požgan, I. Sosič, S. Gobec, A microwave-assisted nucleophilic substitution reaction
436 on a quinoline system: the synthesis of amino analogues of nitroxoline, *Tet Lett*, 53(2012) 1964-7.

437 [29] C. Tarricone, R. Dhavan, J. Peng, L.B. Areces, L.-H. Tsai, A. Musacchio, Structure and regulation of
438 the CDK5-p25nck5a complex, *Mol Cell*, 8(2001) 657-69.

439 [30] M. Mapelli, L. Massimiliano, C. Crovace, M.A. Seeliger, L.H. Tsai, L. Meijer, et al., Mechanism of
440 CDK5/p25 binding by CDK inhibitors, *Journal of medicinal chemistry*, 48(2005) 671-9.

441 [31] L. Kurzawa, M. Pellerano, J.B. Coppolani, M.C. Morris, Fluorescent peptide biosensor for probing
442 the relative abundance of cyclin-dependent kinases in living cells, *PloS one*, 6(2011) e26555.

443 [32] M.C. Morris, J. Depollier, J. Mery, F. Heitz, G. Divita, A peptide carrier for the delivery of
444 biologically active proteins into mammalian cells, *Nat Biotech*, 19(2001) 1173-6.

445 [33] L. Kurzawa, M. Pellerano, M.C. Morris, PEP and CADY-mediated delivery of fluorescent peptides
446 and proteins into living cells, *Biochim Biophys Acta*, 1798(2010) 2274-85.

447 [34] D.M. Shcherbakova, V.V. Verkhusha, Near-infrared fluorescent proteins for multicolor in vivo
448 imaging, *Nature methods*, 10(2013) 751-4.

449 [35] J. Zhang, H. Li, T. Zhou, J. Zhou, K. Herrup, Cdk5 levels oscillate during the neuronal cell cycle:
450 Cdh1 ubiquitination triggers proteasome-dependent degradation during S-phase, *J Biol Chem*,
451 287(2012) 25985-94.

452 [36] T. Kawauchi, Cdk5 regulates multiple cellular events in neural development, function and
453 disease, *Development, growth & differentiation*, 56(2014) 335-48.

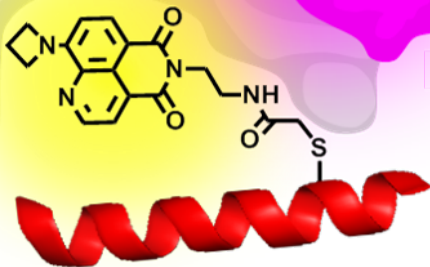
454 [37] D.H. Cho, E.J. Lee, K.J. Kwon, C.Y. Shin, K.H. Song, J.H. Park, et al., Troglitazone, a
455 thiazolidinedione, decreases tau phosphorylation through the inhibition of cyclin-dependent kinase 5
456 activity in SH-SY5Y neuroblastoma cells and primary neurons, *Journal of neurochemistry*, 126(2013)
457 685-95.

458

p25

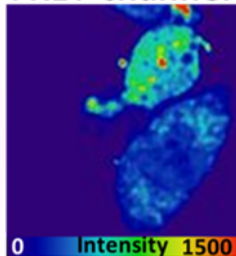
FRET

iRFP670



CDK5 peptide

FRET channel



Lifetime

