

## Two-photon excitation with pico-second fluorescence lifetime imaging to detect nuclear association of flavanols

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**Keywords:** Fluorescence lifetime imaging microscopy, flavanols, epigallocatechin gallate, nuclear binding, histone proteins, multiphoton excitation.

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1

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18 **ABSTRACT**

19 Two-photon excitation enabled for the first time the observation and measurement of

20 excited state fluorescence lifetimes from three flavanols in solution, which were ~ 1.0 ns

21 for catechin and epicatechin, but <45 ps for epigallocatechin gallate (EGCG). The shorter

22 lifetime for EGCG is in line with a lower fluorescence quantum yield of 0.003 compared to  
23 catechin (0.015) and epicatechin (0.018).

24

25 *In vivo* experiments with onion cells demonstrated that tryptophan and quercetin, which  
26 tend to be major contributors of background fluorescence in plant cells, have sufficiently  
27 low cross sections for two-photon excitation at 630 nm and therefore do not interfere with  
28 detection of externally added or endogenous flavanols in *Allium cepa* or *Taxus baccata*  
29 cells. Applying two-photon excitation to flavanols enabled 3-D fluorescence lifetime  
30 imaging microscopy and showed that added EGCG penetrated the whole nucleus of onion  
31 cells. Interestingly, EGCG and catechin showed different lifetime behaviour when bound to  
32 the nucleus: EGCG lifetime increased from <45 to 200 ps, whilst catechin lifetime  
33 decreased from 1.0 ns to 500 ps. Semi-quantitative measurements revealed that the relative  
34 ratios of EGCG concentrations in nucleoli associated vesicles : nucleus : cytoplasm were  
35 *ca.* 100:10:1.

36

37 Solution experiments with catechin, epicatechin and histone proteins provided preliminary  
38 evidence, via the appearance of a second lifetime ( $\tau_2 = 1.9$  to  $3.1$  ns), that both flavanols  
39 may be interacting with histone proteins. We conclude that there is significant nuclear  
40 absorption of flavanols. This advanced imaging using two-photon excitation and  
41 biophysical techniques described here will prove valuable for probing the intracellular  
42 trafficking and functions of flavanols, such as EGCG, which is the major flavanol of green  
43 tea.

44

45 *Keywords:* Fluorescence lifetime imaging microscopy, flavanols, epigallocatechin gallate,  
46 nuclear association, histone proteins, multiphoton.

47

## 48 **1. Introduction**

49 Plants synthesise >4000 different flavonoid compounds, which can be grouped into several  
50 different subgroups. Flavanols (Fig. 1) are an important subgroup that is widespread in  
51 plants and plant foods [1]; they are also precursors of condensed tannins, which are the  
52 fourth largest group of natural plant products after cellulose, hemicellulose, and lignin [2].  
53 These polyphenolic compounds are attracting considerable interest, because diets rich in  
54 fruits and vegetables are associated with improved health and a reduction of age-related  
55 diseases such as cancer, osteoporosis and cardiovascular diseases [3-7]. Flavonoids are  
56 considered to be ‘lifespan essentials’ and recent reviews suggest that their antioxidant  
57 properties alone are unlikely to explain their beneficial effects on human health or their  
58 functions in plants [8-10].

59

60 A consensus is emerging that *in vitro* and *in vivo* experiments need to probe the  
61 bioavailability of these polyphenols and their molecular targets [3,6,8,10-11]. *In vitro*  
62 studies have tended to require 10 to 100-fold higher polyphenol concentrations than are  
63 usually found in mammalian plasma and tissues in order to achieve many of the reported  
64 medicinal effects [5,12]. However, the existence of high-affinity targets for dietary  
65 polyphenols might explain their health-promoting effects and in this context it is pertinent  
66 to examine more closely recent evidence that nuclei from both plant *and* mammalian cells  
67 acted as sinks for flavanols [13-17]. Although the function of these secondary plant

68 metabolites requires further elucidation, evidence is emerging that they may be important in  
69 cell development. For example, loss of flavanols has been linked to defective pollen  
70 development [14]. Different types of flavanol distribution patterns were observed in *Tsuga*  
71 *canadensis* at the sub-nuclear level [13] and the authors questioned whether the epigenetic  
72 code of histones could affect flavanol-chromatin associations. Moreover, Feucht *et al.* [15]  
73 found identical flavanol patterns within different cell lineages in a meristematic plant tissue  
74 and suggested that this could be indicative of a synchronized, transcriptional regulation. In  
75 addition, nuclear flavanol concentrations clearly depended on the season, i.e. during  
76 dormancy they were almost absent but during growth periods relatively high amounts were  
77 observed [18]. The fact that flavanols were associated with both interphase and mitotic  
78 chromosome states posed the question of whether flavanols might be associated with  
79 histones. If this is the case, then this could open a new perspective on genomic regulation.

80

81 This research by Feucht's group made use of the fact that flavanols form a blue  
82 condensation product with dimethylaminocinnamaldehyde (DMACA) [19]. The DMACA  
83 reagent is, however, a relatively aggressive reagent that requires 0.75 M sulfuric acid for  
84 the staining reaction and this could cause some physical damage within the cells. Polster *et*  
85 *al.* [18], therefore, tested the existence of nucleus-bound flavanols with a milder technique,  
86 i.e. laser microdissection and pressure catapulting (LMPC), which separated intact nuclei  
87 from cells and these also stained blue in the subsequent DMACA reaction. Nevertheless,  
88 LMPC causes physical rupture of the cytoplasm that surrounds the nuclei and could have  
89 given rise to an artificial DMACA reaction. Moreover, histological studies with DMACA  
90 cannot distinguish between different flavanols or between flavanol monomers, oligomers or

91 polymers [19]. Techniques are therefore required that can establish the sub-cellular  
92 localisation, and concentrations therein, of flavanols to probe their functionality and  
93 metabolism in plant and mammalian cells.  
94  
95 Nifli *et al.* [29] recently applied confocal fluorescence microscopy to map the intracellular  
96 distribution of a major plant flavonol, i.e. quercetin (Fig. 1), which has a UV absorption  
97  $\lambda_{\text{max}}$  of 372 nm. Quercetin revealed a specific fluorescence (488 nm<sub>ex</sub>/500-540 nm<sub>em</sub>) in the  
98 cellular environment at physiologically relevant concentrations (<5  $\mu\text{M}$ ), which the authors  
99 attributed to non-covalent binding to cellular components. Intracellular tracing of flavanols  
100 ( $\lambda_{\text{max}} \sim 280$  nm; Fig. 1 and Fig. S1) by UV-Vis spectroscopy or confocal fluorescence  
101 microscopy is, however, not possible because plant and mammalian cells contain numerous  
102 compounds which would interfere with the detection of flavanols by giving background  
103 fluorescence signals (termed “auto-fluorescence”). Fig. 2 illustrates the photophysical  
104 processes in a conventional Jablonski diagramme, which depicts one- and two-photon  
105 excitation and various relaxation pathways that are open to the electronic excited state  
106 following photon(s) absorption.

107

108 In fluorescence life-time imaging microscopy (FLIM) both fluorescence intensities and  
109 fluorescence lifetimes of specific compounds can be measured at each pixel in the image  
110 [21,22]. In addition, variations in fluorescence lifetime can provide further image contrast:  
111 lifetime shifts can serve as sensitive probes for detecting molecular interactions and may  
112 yield information on a compound’s environment, such as pH or oxygen concentration [23-  
113 25]. Lifetime,  $\tau$ , is derived from the time-constant of the fluorescence decay (Fig. 2), where

114  $\tau = 1/k_{\text{fluorescence}}$ . FLIM is based on either single- or multi-photon excitation techniques.  
115 Multi-photon excitation with femtosecond lasers offers many advantages for biological  
116 measurements over more conventional single photon excitation [23,26]:  
117 ✓ Excitation with red light that is not directly absorbed by cellular materials.  
118 ✓ Reduced cellular toxicity in biological studies.  
119 ✓ Reduced photo-bleaching.  
120 ✓ Deeper penetration of the near-infrared light into the biological specimen.  
121 ✓ Femtolitre volume excitation.  
122 ✓ A flexible imaging platform that is capable of resolving several (and related)  
123 compounds.  
124 ✓ The ability to deliver UV-equivalent photon energies directly beneath UV absorbing  
125 materials and molecules.  
126 ✓ An ability to perform time-resolved studies due to the short pulsed light source.  
127 In two-photon excitation (2PE) the simultaneous absorption of two lower energy photons  
128 mimics the absorption of a single photon of equivalent higher energy (Fig. 2). Thus, 2PE at  
129 560 nm mimics UV excitation at 280 nm [23,27]. In FLIM ultrafast lasers providing pulse  
130 lengths of the order of 200 femtoseconds ( $200 \times 10^{-15}$  s) enable time-resolved  
131 measurements, which can detect molecular interactions in solution and cells [23-25,28] and  
132 can be used to construct fluorescence life-time maps of a compound's distribution within  
133 viable cells.  
134

135 Here we describe 2PE experiments designed to eliminate any doubts regarding the results  
136 from previous histological studies that employed the DMACA staining reagent. Two-  
137 photon excitation coupled to 3-D fluorescence lifetime imaging microscopy enabled  
138 examination of intact biological tissues and highly localised, non-destructive and selective  
139 detection of flavanols. The fluorescence behaviour of three flavanols, catechin, epicatechin  
140 and epigallocatechin gallate (EGCG) (Fig. 1), was measured first in model solution systems  
141 and then in two natural cell systems, onion epidermis cells and *Taxus* pollen mother cells.  
142 These flavanols were chosen because they are widespread in plants and are also  
143 bioavailable and bioactive in several *in vitro* and *in vivo* mammalian cell systems  
144 [1,6,9,29]. Solution phase studies were first used to optimise and measure spectroscopic  
145 shifts and lifetime changes of free flavanols *versus* flavanols bound to DNA or histone  
146 proteins at normal physiological pH values. The optimised spectroscopic parameters were  
147 then applied to probe the intra-cellular location of externally added flavanols in *Allium cepa*  
148 cells and of endogenous flavanols in *Taxus baccata* cells. The same plant models had been  
149 tested previously with the DMACA staining reagent [14,30].

150

## 151 **2. Methods and materials**

### 152 *2.1. Reagents*

153 The following reagents were purchased from Sigma-Aldrich Company Ltd, UK:  
154 (+)-catechin (98%), (-)-epicatechin (90%), (-)-epigallocatechin gallate (95%; EGCG), tris-  
155 (hydroxymethyl)amino methane (Tris), K<sub>2</sub>HPO<sub>4</sub> (ACS reagent, (≥ 98%), KH<sub>2</sub>PO<sub>4</sub> (ACS  
156 reagent, (≥ 99%), DNA from calf thymus and Histone type II-A. Histone was supplied by  
157 Roche Diagnostics Ltd, UK. Histone sulphate from calf thymus was purchased from Fluka



158 (Sigma-Aldrich Chemie, Steinheim, Germany; Polster *et al.*, 2003). Ethanol (LiChrosolv, ≥  
159 99.9%) was purchased from VWR-Merck, UK.

160

161 Tris buffers (0.1 M) were prepared and adjusted to pH 7.0 and 8.0 with HCl and phosphate  
162 buffers (0.1 M) at pH 5.8, 7.1 and 8.2 were prepared using K<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> as  
163 described [16].

164

## 165 2.2. Calculation of relative fluorescence quantum yields of the flavanols

166 Flavanols were dissolved in methanol to yield 0.01 M stock solutions. Subsequent dilutions  
167 for 20 and 40 μM flavanol concentrations were made with sodium phosphate buffer (pH  
168 7.4, 0.1 M, 0.05% sodium azide). These were placed in a 10 mm quartz Suprasil  
169 fluorescence cuvette (Hellma, Germany) and UV-Vis spectra were first recorded from 190  
170 to 500 nm using a Helios β spectrophotometer (Spectronic Unicam, U.K.). Then  
171 fluorescence spectra were recorded using a luminescence LS-55 spectrometer (Perkin  
172 Elmer, U.K.) from 290 to 530 nm with excitation at 295 nm under continuous stirring. The  
173 excitation and emission slits were both set to 5 nm and scanning speed was 200 nm min<sup>-1</sup>.  
174 All experiments were carried out at 37 °C. The literature reported a quantum yield of 0.12  
175 for tryptophan (Trp) at 270 nm (website) and we confirmed this for 295 nm. Therefore, the  
176 quantum yields of flavanols (Flav) were calculated relative to tryptophan using the  
177 integrated area between 300 and 530 nm under the fluorescence spectra [31-33] according  
178 to:

179 
$$\text{Absorption at 295 nm (Trp)} * 0.12 * \text{Fluorescence (Flav)}$$

180 
$$\text{Quantum yield}_{(\text{Flav})} = \frac{\text{Absorption at 295 nm (Trp)} * 0.12 * \text{Fluorescence (Flav)}}{\text{Absorption at 295 nm (Flav)} * \text{Fluorescence (Trp)}}$$

181 Absorption at 295 nm (Flav) \* 0.12 \* Fluorescence (Trp)

182

### 183 2.3. Flavanol solutions

184 Flavanols were dissolved in ethanol (~10 mM) and prepared fresh on a daily basis. Just  
185 before measuring the fluorescence lifetimes, aliquots (10 µL) were removed and diluted  
186 with buffer, DNA or histone protein solutions (90 and 40 µL) to obtain flavanol  
187 concentrations between 1 and 2 mM.

188

189 DNA (0.6 mg) was dissolved in Tris buffer (pH 8.0; 30 mL) overnight at 4 °C. Sigma  
190 histone (5.9 mg) was dissolved in Tris buffer (pH 7.0 and 8.0; 2.85 mL). Ethanol (98 µL)  
191 was added to the pH 8.0 buffer to facilitate dissolution. Histone sulphate (0.8 mg) was  
192 dissolved in Tris buffer (pH 7.0 and 8.0) according to Polster *et al.* [16]. The supernatants  
193 were used after centrifugation. Roche histone (1.0 mg) was dissolved in Tris buffer (pH 7.0  
194 and 8.0; 500 µL) and ethanol (10 µL).

195

### 196 2.4. Plant samples

197 The thin adaxial epidermis from onion (*Allium cepa*) bulb scale was removed, cut into 2  
198 cm<sup>2</sup> pieces and incubated with aqueous catechin or EGCG solutions (1 mM; 20 mL) for up  
199 to 8 h [30].

200

201 Male cones from yew (*Taxus baccata*) were harvested on 5<sup>th</sup> October 2008. The eight cover  
202 leaves were removed and the yellow anthers were gently squeezed with tweezers in order to  
203 release the mother pollen cells. Preliminary experiments revealed that these cells stained

204 dark blue with the DMACA reagent (10 mg DMACA dissolved in 1 mL of 0.75 M H<sub>2</sub>SO<sub>4</sub>)  
205 [30].

206

### 207 *2.5. Multiphoton microscopy*

208 The set up used in this study has been previously described [23]. Briefly, a custom built  
209 two-photon microscope was constructed using scanning XY galvanometers (GSI Lumonics  
210 Ltd). A diode-pumped (Verdi V18) titanium sapphire (Mira F900) operating at 700-980 nm  
211 generated laser light at a wavelength of  $585 \pm 2$  nm and was used for the solution studies  
212 and at  $630 \pm 2$  nm for the plant cell studies through an optical parametric oscillator (OPO,  
213 APE-Coherent GmbH, Berlin, Germany) operating at 180 fs pulses at 75 MHz. The pulse  
214 width was maintained using a femto control unit (APE Coherent GmbH). The laser beam  
215 was focused to a diffraction-limited spot using a water-immersion ultraviolet corrected  
216 objective (Nikon VC x60, NA 1.2) and specimens were illuminated at the microscope stage  
217 of a modified Nikon TE2000-U with UV transmitting optics. Fluorescence emission was  
218 collected without descanning, bypassing the scanning system, and passed through a  $340 \pm$   
219 20 nm interference filter (U340, Comar Instruments, Cambridge, UK). Emission  
220 fluorescence was detected using an external fast microchannel plate photomultiplier tube  
221 (Hamamatus R3809U-50) and recorded using a Time-Correlated Single Photon Counting  
222 (TCSPC) PC module SPC830 (Becker and Hickl GmbH, Berlin, Germany). Fluorescence  
223 lifetime image microscopy was performed by synchronising the XY galvanometer positions  
224 with the fluorescence decay. The X,Y galvanometers were raster scanned at 1 ms or 2 ms  
225 per pixel for 128 x 128 or 256 x 256 image size, respectively, giving a 33 sec per image

226 frame. The presented images were three accumulations to allow for enough photon counts  
227 per channel for the data analysis.

228

## 229 2.6. *Image analysis*

230 Steady state grey scale images (8 bit, up to 256 x 256 pixels) are produced by binning all  
231 decay photons as a single channel. Fluorescence lifetime images were obtained for control  
232 cells and flavanol-loaded cells by analysing the decay at individual pixels using a single or  
233 double exponential curve fitting (SPCImage 2.94 analysis software Becker and Hickl). A  
234 thresholding function within the FLIM analysis software ensured that noncorrelating  
235 photons leading to background noise arriving at the detector were not included in the  
236 analysis. Single point decay analysis was carried out without binning while FLIM was  
237 analysed with a maximum of 2 binning.

238

## 239 **3. Results and discussion**

### 240 3.1. *Flavanol fluorescence lifetimes in aqueous solutions*

241 It is well known that flavanols oxidise readily in alkaline pH [4], therefore lifetimes were  
242 first examined at pH values ranging from 5.8 - 8.2. Fig. 3 shows that the fluorescence  
243 lifetime,  $\tau$ , of catechin (2 mM catechin solution in 0.1 M phosphate buffer) was relatively  
244 stable between pH 5.8 and 7.1:  $\tau$  was 1.0 ns at the start and 0.9 ns after 20 min. However, at  
245 pH 8.2 the lifetime changed from 1.0 to 0.7 ns within 20 minutes. When the same  
246 measurements were conducted under a nitrogen blanket, lifetime reduction was kept to 9%  
247 over a 30 min period and this agrees with Sang *et al.* [34] who found that flavanols were  
248 not oxidised under nitrogen. Therefore, all subsequent solution measurements were

249 determined immediately after mixing the solutions, i.e. within 30 seconds. Fig. 3 also  
250 shows that pH *per se* had no effect on catechin lifetimes:  $\tau$  of catechin was  $\sim 1.0$  ns at pH  
251 5.8, 7.1 and 8.2. The reduction in  $\tau$  values can also not be ascribed to sample concentration  
252 or the presence of non-interacting or energy transfer products, as the excited state lifetime  
253 is independent of both of these.

254

255 The natural lifetime of catechin in solution in the absence of oxygen is  $\sim 1.1$  ns (Fig. 3).  
256 This reduces, via quenching, as expected in the presence of dissolved oxygen ( $7.6 \text{ mg}\cdot\text{L}^{-1}$ )  
257 at room temperature and pressure to  $\sim 1$  ns. It is worth noting that at high oxygen  
258 concentrations, 30 mM, the quenched lifetime observed will be as expected taking into  
259 account diffusion control rate. Therefore the subsequent change in lifetime (Fig. 3) (to  $\sim 0.7$   
260 ns after 20 min in oxygen) is very likely due to the formation of a deprotonated or oxidised  
261 product as the OH groups in the B-ring are particularly susceptible to deprotonation and  
262 therefore oxidation at alkaline pH [35]. It is interesting to note that the reduced lifetime  
263 fitted well to a single exponential decay, again indicating a single fluorescent molecular  
264 species is present and favouring the observed decreases in lifetime results from either a  
265 photoproduct, which also fluoresces, or an oxygen quenched process. Further studies using  
266 high performance liquid chromatography may help identify these oxidised products.

267

268 Importantly, Fig. 4 shows that flavanols had different fluorescent decay curves.

269 Fluorescence lifetimes of catechin and epicatechin were similar (1.0 and 1.1 ns,

270 respectively). However, in the case of EGCG at pH 8.1 (2 mM flavanol solutions in 0.1 M

271 phosphate buffer) the lifetime was found to be within the instrument response function and  
272 Fig. 4 shows only the characteristics of the fast micro-channel plate (<45 ps). Ultrafast time-  
273 resolved Kerr gated fluorescence spectroscopy will be needed to resolve the EGCG lifetime  
274 in the future. EGCG differs from catechin and epicatechin by the presence of a galloyl  
275 group at C-3 (Fig. 1). The lifetime of the excited state is given by the sum of the different  
276 competing relaxation processes, which include fluorescence, non-radiative decay,  
277 intersystem crossing and chemical reaction as illustrated in Fig. 2. The shorter lifetime for  
278 EGCG is most likely due to the presence of additional phenolic groups, which would be  
279 expected to enhance the solvation effects and which in turn would influence the non-  
280 radiative decay processes. These extra phenolic groups also enhance its antioxidant  
281 properties [36] and this presumably makes it more susceptible to oxidation. Indeed, the  
282 fluorescence quantum yield of EGCG is much lower than that of catechin or epicatechin  
283 (Table 1) suggesting that the non-radiative rate ( $k_{IVR}$ ; Fig. 2) dominates in the relaxation of  
284 the electronic excited state.

285

286 At pH 8, epicatechin also had a two-component fluorescence decay lifetime (see footnote in  
287 Supplementary Table). The exact physical origin of the bi-exponential lifetime is unknown.  
288 However, it is not uncommon for fluorophores in complex cellular environments to  
289 demonstrate multiple decay times as seen in Table 2. Different decay times represent  
290 differing physical influences that the nascent electronic excited states are subjected to and  
291 consequently may lead to differences in the efficiency of the energy loss process and return  
292 to the ground state. The fact that we see a bi-exponential decay indicates that the flavanols  
293 find themselves in two differing states and/or two different environments; for example free

294 and bound forms (Supplementary Table). Further investigations studying the ultrafast  
295 dynamics will be needed to help explain these differences and/or whether diastereoisomers  
296 such as catechin and epicatechin have different fluorescence properties.

297

## 298 *3.2. Fluorescence lifetime imaging microscopy*

### 299 *3.2.1. Control experiments with onion cells*

300 The experimental conditions developed above for flavanol solutions were applied initially  
301 to onion root cells (tissue soaked in water for 5 h; and followed by two-photon excitation at  
302 585 nm). Lifetime decay curves, at several different points in the cells, could be fitted to a  
303 single exponential decay giving a  $\tau$  value between 2.3 and 2.6 ns ( $\chi^2 = 1.05$ ). It is highly  
304 likely, however, that under these excitation conditions the emission and lifetime values are  
305 mainly due to auto-fluorescence contributions from tryptophan [23]. In order to avoid  
306 significant background fluorescence signals from other cellular materials, in particular  
307 aromatic amino acids, e.g. tryptophan, when using UV excitation at 290 nm (equivalent to  
308 580 nm 2PE excitation) the 2PE excitation wavelength was shifted to 630 nm, which has  
309 been shown to give little background interference [23]. Control experiments were then  
310 carried out without added flavanols, i.e. in the presence of just water, in order to  
311 substantiate that the fluorescence was due to flavanols. The onion sample without added  
312 flavanol showed only weak auto-fluorescence and a  $\tau$  value of 0.8 ns ( $\chi^2 = 1.60$ ) (Fig. 5c)  
313 confirming that our FLIM measurements were tracking the flavanol presence in cells (see  
314 Section 3.2.2. below). Whole onions are known for their high quercetin concentration (Fig.  
315 1) [6], but given the low photon count, we can conclude that neither tryptophan nor

316 quercetin interfered with flavanol detection,  $\lambda_{em}$ , at  $340 \pm 20$  nm if 2PE with  $\lambda_{ex}$  was 630  
317 nm.

318

### 319 3.2.2. Absorption of flavanols by onion nuclei

320 Fig. 5a and 5b show fluorescence lifetime maps of cells in an onion epidermis, which had  
321 been soaked in 1 mM aqueous flavanol solutions. Following absorption of catechin or  
322 EGCG, the fluorescing nuclei and several bright, small spots of  $\sim 2$  to  $7 \mu\text{m}$  were clearly  
323 visible to a much greater extent than the surrounding cell matrix. Careful analysis of Fig. 5b  
324 showed a bright spot of  $4 \mu\text{m}$  diameter. It is known that inactive nuclei possess very small  
325 nucleoli of the order of  $\sim 1 \mu\text{m}$  [37]. The observed spot is too large to be a nucleolus, we  
326 therefore propose that the bright spot was a clustering of perinucleolar organiser regions  
327 (NORs) [38]. NORs tend to surround the nucleoli and strongly absorb flavanols [13].

328

329 This study demonstrated that FLIM combined with 2PE at 630 nm enabled *in vivo*  
330 detection of both catechin and EGCG and avoided interference by tryptophan or quercetin,  
331 as the control showed hardly any fluorescence (Fig. 5c). We have previously shown that  
332 there is negligible excitation of cellular auto-fluorescence, particular from tryptophan,  
333 following multiphoton excitation at 630 nm [23]. Although tryptophan may be excited by  
334 multi-photon treatment at 590 nm, which is equivalent to single photon excitation (1PE) at  
335 295 nm, this diminishes by a factor of 10 at 630 nm. Furthermore, the excited state lifetime  
336 of tryptophan ( $\sim 3$  ns) is significantly different to that of the flavanols investigated here.  
337 These findings, therefore, provided clear and unequivocal evidence for nuclear flavanol  
338 absorption. Since the excited state lifetime may be influenced by the environment of the



339 flavanols, the colour trend seen in the FLIM images (Fig. 5a,b) may be due to slight  
340 differences in the environment of the absorbed flavanols. A series of  $z$  axis images taken  
341 through a cell revealed that EGCG was detectable throughout the nucleus and not just at the  
342 surface (Video Clip S1). EGCG appeared to be concentrated in the NORs; relative  
343 proportion of EGCG photon counts were 1 to 3 (cytoplasm) : 10 (nucleus) : 100 to 150  
344 (NORs) (*data not shown*).

345

### 346 3.3. FLIM lifetimes of bound versus free flavanols in solution

347 Fluorescence decay curves of nucleus-bound catechin were best fitted to two components,  
348 i.e.  $\tau_1 = 0.5$  ns (77.5%) and  $\tau_2 = 2.7$  ns (22.5%;  $\chi^2$  of 1.04) (Table 2). It is unlikely that  $\tau_2$   
349 emanates from tryptophan as the same experiments with EGCG fitted to a single  
350 component decay with an average  $\tau$  of  $0.25 \pm 0.05$  ns (Table 2). The increase in EGCG  
351 lifetime from  $<0.045$  ns in solution (Fig. 4) to 0.25 ns in the nucleus is a reverse of the  
352 trend seen for catechin, which showed a lifetime of  $\sim 1$  ns in solution and 0.5 ns in the  
353 nucleus.

354

355 The effect of nuclear association generating different lifetimes is given in Table 2 and was  
356 recorded when Fig. 5 was taken. Taken together, these observations suggest that the two  
357 flavanols (catechin and EGCG) may differ in their interaction mechanisms with nuclear  
358 components. The lowering of a lifetime indicates either an enhanced non-radiative decay  
359 (through for example formation of hydrogen bonds) [31] or possibly self-association which  
360 has been reported for catechin [39]. A decrease in lifetimes upon cellular absorption has  
361 also been reported for 5-hydroxytryptophan and was attributed to self-quenching or

362 environmental effects [23]. Further research will be needed to establish whether oxidation  
363 during the cellular absorption experiment could have contributed to the shorter catechin  
364 lifetime (Fig. 3) and whether oxidation would have increased EGCG fluorescence lifetime.  
365 It seems, however, more likely that this contrasting lifetime behaviour is indicative of  
366 different interaction mechanisms.

367

#### 368 *3.4. Endogenous flavanols in Taxus baccata*

369 The same 2PE experimental conditions were then applied to pollen mother cells which had  
370 been isolated from microspores of male *Taxus baccata* cones. According to Feucht *et al.*  
371 [14] late tetrads and early microspores possess endogenous catechin and epicatechin. We  
372 observed, however, fluorescence lifetimes, which could be fitted to single component  
373 decays with  $\tau$  of 0.2 ns and which resembled EGCG, rather than catechin or epicatechin  
374 (Table 2). Interestingly, the photon count of the signal to noise ratio from endogenous  
375 flavanols in the *Taxus* cells was not dis-similar to onion cells soaked in a 1 mM EGCG  
376 solution. Younger cones at the tip of the *Taxus baccata* twig yielded twice as many photons  
377 compared to slightly more mature cones from further along the twig. This finding agrees  
378 with previous observations [14,15] that nuclear DMACA staining for flavanols was most  
379 intense during high cell activity, e.g. in mitotic and stem cells. Interestingly, it also  
380 coincides with observations of higher EGCG concentrations in foetal than maternal plasma  
381 of rats: absorbed catechins were found in the brain, eye, heart, lung, kidney, liver and  
382 placenta of fetal organs [40].

383

#### 384 *3.5. Flavanol fluorescence lifetimes in the presence of DNA or histone proteins*

385 The fact that flavanols bind to the nucleus raises an important question: which nuclear  
386 components act as the binding sites? Several previous studies demonstrated that DNA  
387 interacts with planar flavonoids, such as flavonols and anthocyanidins, and depending on  
388 the experimental conditions, these interactions were either weak or led to intercalation [41].  
389 However, flavanols are *not* planar and may therefore not be able to intercalate with DNA.  
390 We, therefore, explored fluorescence lifetime behaviour of flavanols in the presence of  
391 DNA. Addition of DNA had no effect on catechin or epicatechin lifetimes in aqueous  
392 solutions (2 mM; pH 8 in Tris buffer; data not shown). This agrees with results from UV-  
393 Vis spectroscopic titrations which also found that DNA did not interact with catechin or  
394 EGCG in 0.1 M Tris at pH 7.4 or 8.0 [16].

395

396 However, Polster *et al.* [16] reported that histone proteins might be the nuclear targets for  
397 catechin and EGCG. Using UV-Vis titration experiments, they showed that both flavanols  
398 bound to histone sulphate and interactions were more pronounced at pH 8.0 than 7.4 in Tris  
399 buffer. Since these titration experiments required approximately 1 h in total [42], it could be  
400 argued that this might be sufficient time for oxidative reactions and artefact formation to  
401 occur especially at higher pH values as determined in Fig. 3. The present fluorescence  
402 lifetime measurements were, however, made within 30 s of mixing the flavanol and histone  
403 solutions (note: the fluorescence lifetime experiments were also done in Tris buffer, as the  
404 UV-Vis titrations revealed that histone sulphate showed a less pronounced interaction with  
405 catechin in phosphate than Tris buffer [16]).

406

407 The lifetimes of catechin and epicatechin in the presence of histone proteins were  
408 investigated at two concentrations (1.1 and 1.9 mM) and pH (7 and 8) (Supplementary  
409 Table). Given the short lifetimes recorded and the errors in fitting bi-exponential decays,  
410 the data for the different flavanols need careful interpretation. At this stage we are unable to  
411 clearly identify whether or not histones bind to flavanols, which would be expected to be  
412 shown by a change in lifetime (i.e. due to quenching). From other work it is, however, also  
413 clear that histone preparations differ in their ability to associate with flavanols [13] and,  
414 therefore, further fluorescence studies will be needed. Nevertheless, these initial findings  
415 demonstrate the potential power of studying flavanol–histone interactions by fluorescence  
416 methods. It is now also evident that much fundamental work is needed for characterising  
417 how the chemical environment (including pH and oxygen concentration) influence  
418 fluorescence lifetimes, quantum yields and spectra of flavanols. With regard to pH, the  $pK_a$   
419 values of catechin, for example, are *ca.* 8.6 and 9.4 [43] and thus at pH 8 three different  
420 protolyte species exist for catechin:  $BH_2$ ,  $BH^-$ , and  $B^{2-}$ . The fluorescence behaviour of each  
421 of these species will need to be understood and only through such careful measurements  
422 can these types of fluorescence measurements provide the much needed tool for elucidating  
423 the interactions between flavanols, histones and DNA.

424

### 425 *3.6. Possible role of nuclear flavanols beyond an antioxidant function*

426 Nuclear absorption has been reported not only for flavanols [13] but also for some other  
427 flavonoids. *Arabidopsis thaliana* nuclei absorb flavonols [44,45], *Drosophila* follicle nuclei  
428 absorb quercetin [46] and *Flaveria chloraefolia* nuclei absorb sulfonated flavanols [47]. It  
429 used to be widely accepted that the major function of polyphenols such as flavonoids was

430 to protect DNA against UV damage and oxidative stress, but this has now been questioned  
431 [8,10]. Instead they were shown recently also to affect cell signalling and gene expression  
432 [48,49]. Flavanols are involved in the transcriptional activation of genes and modulation of  
433 epigenetic changes [36,50].

434

435 Whilst several dietary flavonols and the green tea flavanol, EGCG, have been implicated in  
436 interacting and protecting DNA against damage [41,51, 52], the fact that they inhibit DNA  
437 methyltransferases *in vitro* and *in vivo* at the  $\mu$ molar to sub- $\mu$ molar level is potentially more  
438 important for their effects on health [5,7,29,48,53]. Moreover, EGCG was also a potent  
439 inhibitor of histone acetyltransferase [50]. Histone acetylation has previously been shown  
440 to affect flavanol association [13,16] and is known to alter the chromatin structure, which in  
441 turn has been linked to the transcriptional activation of genes [15]. Both processes, DNA  
442 methylation and histone acetylation, are involved in epigenetic changes [54]. Indeed,  
443 Yamada *et al.* [53] concluded that EGCG may have inhibitory effects on the epigenetic  
444 changes that occur during carcinogenesis and aging. Whilst we found no evidence for  
445 interactions between flavanols and DNA, the results presented here in terms of flavanol  
446 association and penetration through the nucleus do not rule out the possibility that histones  
447 may be a target for EGCG, catechin and epicatechin. Solution phase ultrafast structure and  
448 dynamics studies such as time resolved infra-red (IR) or time resolved 2-dimensional IR  
449 will be needed to probe the origin of the bi-exponential lifetimes, which differed between  
450 the three flavanols. Such time resolved spectroscopic techniques will indicate the functional  
451 groups responsible for the fast dynamics that differ amongst these flavanols.

452

453 3.7. *Future prospects*

454 Given the recent discoveries identifying that flavanols may well be involved in epigenetic  
455 changes, highly sensitive techniques will be needed to trace their uptake and trafficking at  
456 the sub-cellular and sub-nuclear level and at physiologically relevant concentrations. Our  
457 results suggest that not all flavanols will interact via the same molecular mechanism and  
458 this will require new techniques with sufficient specificity and sensitivity. New  
459 developments in fluorescence lifetime imaging techniques and ultra-fast spectroscopy, as  
460 demonstrated here, may hold the key and pave the way for studying their functions and  
461 synthesis in plant cells. The trafficking, uptake and subcellular localisation of flavanols is  
462 of acute interest also for current research on tannin synthesis in plants [55]. Unravelling this  
463 last hurdle of flavonoid biosynthesis, storage and release would facilitate the development  
464 of new plant varieties with tannin compositions that can offer enhanced biological activities  
465 for nutrition and health [11].

466 Such analytical developments will facilitate new types of biological experiments that can  
467 test how these compounds, when present in plant foods, can impact on mammalian cells  
468 and health. Although FLIM is a new technique to both mammalian and plant cell biologists  
469 alike, its application is growing rapidly [23-26,28], particularly in protein-protein  
470 interactions that involve energy transfer processes. However, this is the first study to report  
471 FLIM for other plant components and as the technique becomes more readily available, its  
472 impact can only grow. This study has shown that relatively small changes in flavanol  
473 structures (EGCG *versus* catechin or epicatechin; Fig. 1) lead to measurable changes in  
474 lifetime behaviour in the free and bound states. As plants synthesise several types of  
475 flavonoids, that vary in oxidation and substitution patterns [1], it is expected that other

476 flavonoid compounds will be detectable using different combinations of excitation and  
477 emission wavelengths.

478

#### 479 **4. Conclusions**

480 In conclusion, 2-photon excitation at 585 and 630 nm has enabled for the first time the  
481 measurement of fluorescence lifetimes from three flavanols, catechin, epicatechin and  
482 EGCG, in solution and *in vivo*. Lifetimes ranging from <45 ps to 1 ns in solution have been  
483 determined. *In vivo* experiments with onion cells demonstrated that tryptophan and  
484 quercetin have sufficiently low absorbance at 630 nm and this allowed the detection of  
485 externally added and endogenous flavanols within *Allium cepa* and *Taxus baccata* cells.  
486 Interestingly, fluorescence decay curves of catechin and EGCG differed markedly both in  
487 solution and when bound at the nucleus. This fact could be used in the future for selectively  
488 tracing the different flavanols *in vivo*. Furthermore, this work demonstrates how the  
489 application of fluorescence lifetime technology may be used to investigate the way  
490 flavanols interact with individual cellular components. We also conclude that flavanols are  
491 absorbed by cell nuclei and this provides new research challenges with regard to their  
492 intracellular functions.

493

494 Semi-quantitative measurements revealed that the relative ratios of EGCG concentrations in  
495 perinucleolar organiser regions : nucleus : cytoplasm were approximately 100:10:1.

496 Moreover, 3-D FLIM showed that externally added EGCG penetrated the whole nucleus of  
497 onion cells and was not just absorbed on the surface. The FLIM technique described here  
498 proved therefore a significant advance to DMACA staining and is capable of providing

499 quantitative biophysical information to probe the intra-cellular functions of flavanols such  
500 as EGCG, which is the major flavanol of green tea.

501

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506

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- 596

597 **Legend to Figures**

598

599 **Fig. 1:**

600 Structures of three flavanols, catechin (**1**), epicatechin (**2**), and epigallocatechin gallate (**3**),  
601 and one flavonol, quercetin (**4**) (*note*: A, B, C denote the flavonoid rings).

602

603 **Fig. 2:**

604 The Jablonski diagramme depicting the energy levels of a molecule.  $S_0$  represents the  
605 ground singlet states,  $S_1$ ,  $S_2$  the excited singlet states;  $T_1$  the triplet excited states. Electronic  
606 levels are subdivided into vibrational levels ( $v_1, v_2 \dots v_n$ ). IC indicates internal conversion,  
607  $k_{\text{fluorescence}}$ : rate of fluorescence leading from  $S_1$  ( $v_1 = 0$ ) to  $S_0$  ( $v_1$  or  $v_n$ ),  $k_{\text{IVR}}$ : intramolecular  
608 vibrational relaxation,  $k_{\text{ISC}}$ : rate of intersystem crossing and  $k_{\text{quench}}$ : rate of reaction with  
609 other molecules, chemical or energy transfer.

610

611 **Fig. 3:**

612 Time course of fluorescence lifetimes (ns) of catechin (2 mM) in 0.1 M phosphate buffer in  
613 air or nitrogen atmospheres at pH 5.8, 7.1 and 8.2 ( $\lambda_{\text{ex}} = 585$  nm).

614

615 **Fig. 4:**

616 Fluorescence decay curves of catechin, epicatechin and epigallocatechin gallate (EGCG)  
617 solutions (2 mM) in 0.1 M phosphate buffer at pH 8.1 ( $\lambda_{\text{ex}} = 585$  nm).

618

619 **Fig. 5:**

620 Fluorescence lifetime images ( $\lambda_{\text{ex}} = 630 \text{ nm}$ ) of a cell from an onion epidermis soaked in 1  
621 mM aqueous solutions of a) catechin ( $\tau_1 = 0.4 \text{ ns}$  (82%),  $\tau_2 = 2.6 \text{ ns}$  (18%)), b)  
622 epigallocatechin gallate ( $\tau = 0.2 \text{ ns}$ ) and c) control in water without added flavanol. Image  
623 (A) shows a steady state image of the total emission lifetimes and image (B) shows the  
624 analysed fluorescence excited state map. The distribution of fluorescence lifetimes in (B) is  
625 illustrated in image (C), where the vertical axis represents the frequency and the horizontal  
626 axis represents lifetime in pico-seconds. (*Note*: control nucleus shows hardly any  
627 fluorescence in Fig. 5c).

628

### 629 **Supporting information**

630 Additional Supporting Information may be found in the online version of this article:

631 **Supplementary Table:** Fluorescence lifetimes (ns) of flavanols in the presence of different  
632 histones (Tris buffers, pH 7 and 8). Pre-exponential factors are shown in brackets.

633

634 **Fig. S1.** UV-Vis spectra of catechin, epicatechin and epigallocatechin gallate recorded from  
635 200 to 595 nm.

636

637 **Video Clip S1.** 3D stack of multiphoton excited ( $\lambda_{\text{ex}} = 630 \text{ nm}$ ) fluorescence image from an  
638 onion cell epidermis soaked in a 1mM aqueous solution of epigallocatechin gallate. Images  
639 were recorded at 0.5 - 2.0  $\mu\text{m}$  slices.

640

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643 should be directed to the corresponding author for the article.

644  
645

646 **Table 1**

647 Fluorescence quantum yields of catechin, epicatechin and epigallocatechin gallate (EGCG)  
648 in methanol at 37 °C.

649

<b>Compound</b>	<b>Quantum yield<sup>a</sup></b>
Catechin	0.018
Epicatechin	0.015
EGCG	0.003

650

651 <sup>a</sup> Estimated accuracy = ±16%

652



653 **Table 2**

654 Fluorescence lifetimes (ns) and pre-exponential factors ( $A_1$  and  $A_2$ ) of externally added  
 655 flavanols, which were absorbed by onion epidermis, and endogenous flavanols in *Taxus*  
 656 *baccata* male cones ( $\pm$  standard deviations).  
 657

Sample	$\tau_1$ (ns)	$A_1$ %	$\tau_2$ (ns)	$A_2$ %
Onion epidermis:				
control in water	0.84 <sup>a</sup>	100		
Onion epidermis:				
+ catechin	0.5 $\pm$ 0.04	77.5 $\pm$ 6.92	2.7 $\pm$ 0.19	22.5 $\pm$ 6.92
+ epigallocatechin gallate (EGCG)	0.25 $\pm$ 0.05	99.2 $\pm$ 1.13		
<i>Taxus baccata</i> male cones:				
in water	0.2 $\pm$ 0.03	93.4 $\pm$ 6.46	0.5 $\pm$ 0.2	6.6 $\pm$ 6.46

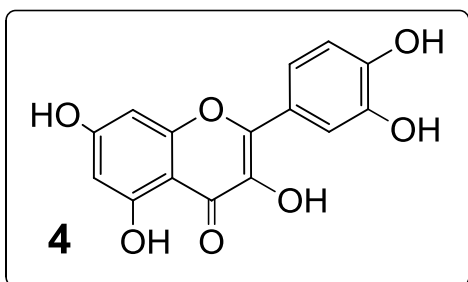
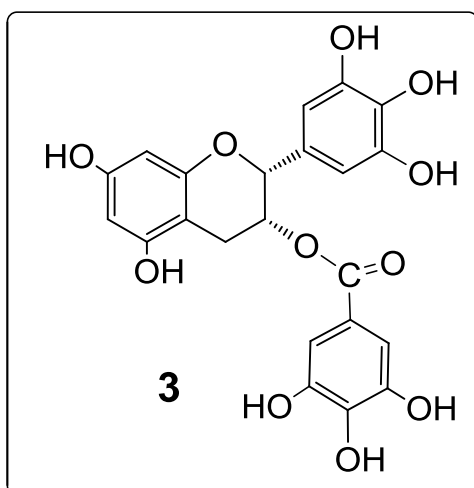
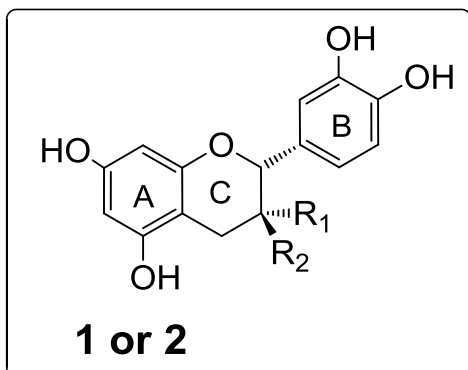
658

659 <sup>a</sup> The control had a very low photon count in the absence of externally added flavanols and  
 660 the data were quite noisy (see Fig. 5c), therefore it was not possible to obtain a standard  
 661 deviation of the background lifetime.

662

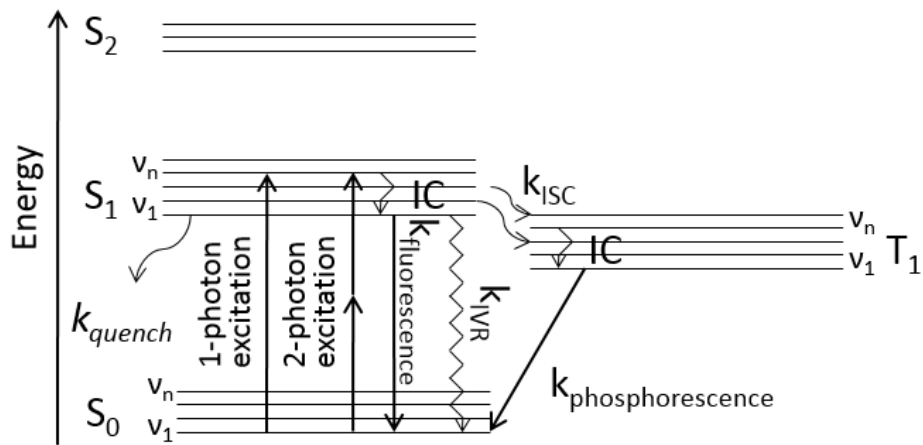
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664 **Figure 1:**  
665

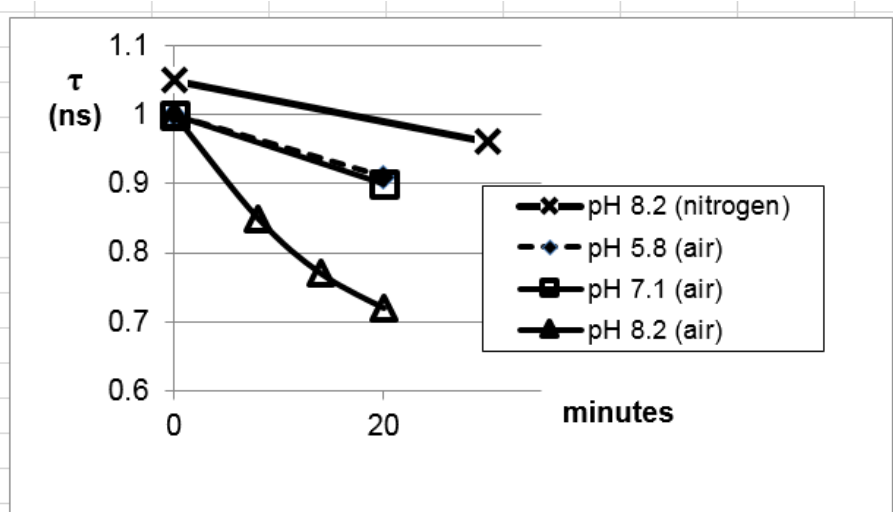


666  
667  
668

669 Fig 2  
670

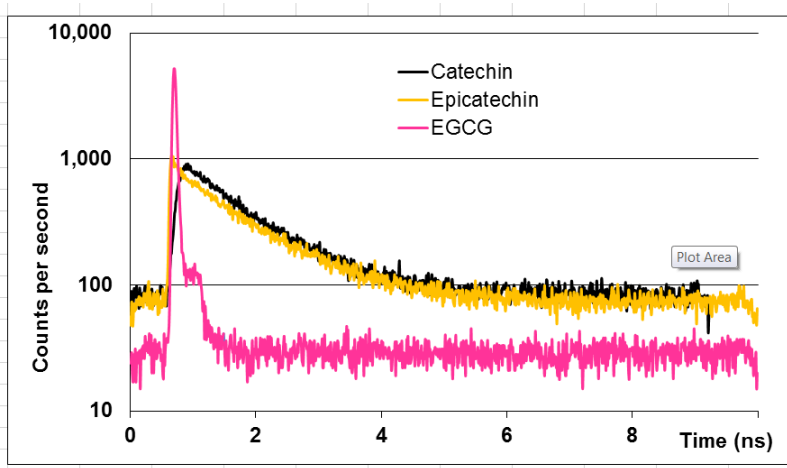


671  
672 Fig 3  
673  
674



675  
676

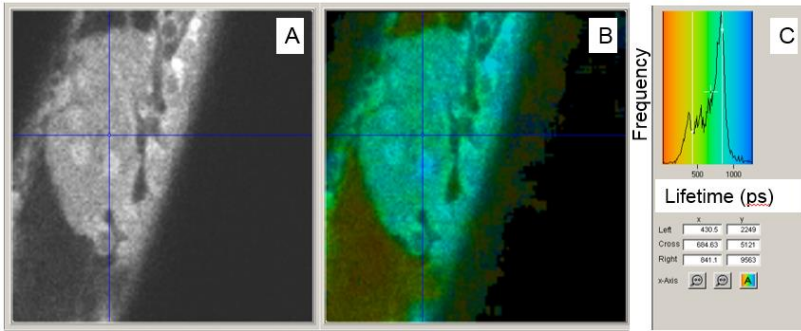
677 Fig 4



678

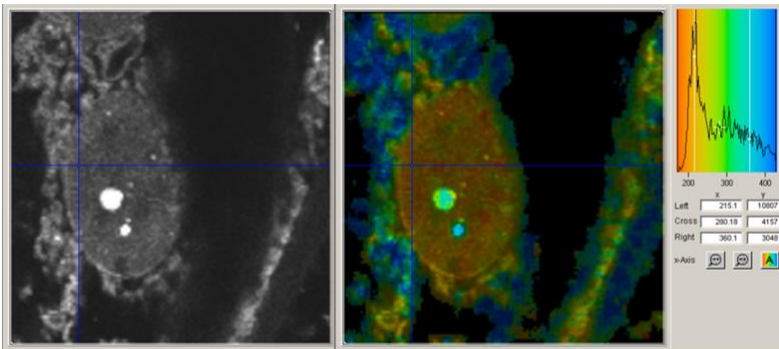
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680 Fig 5a



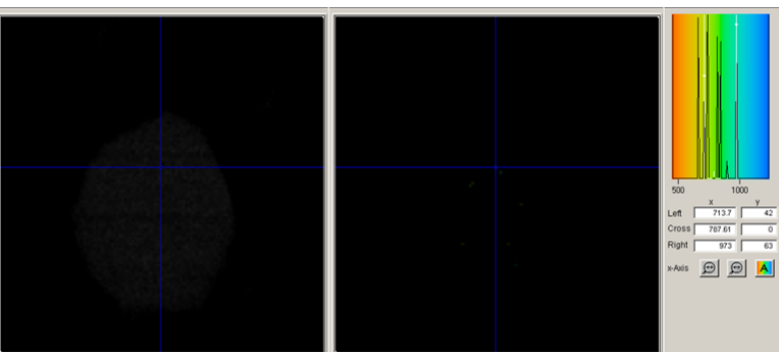
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682 Fig 5 b



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684 Fig 5c



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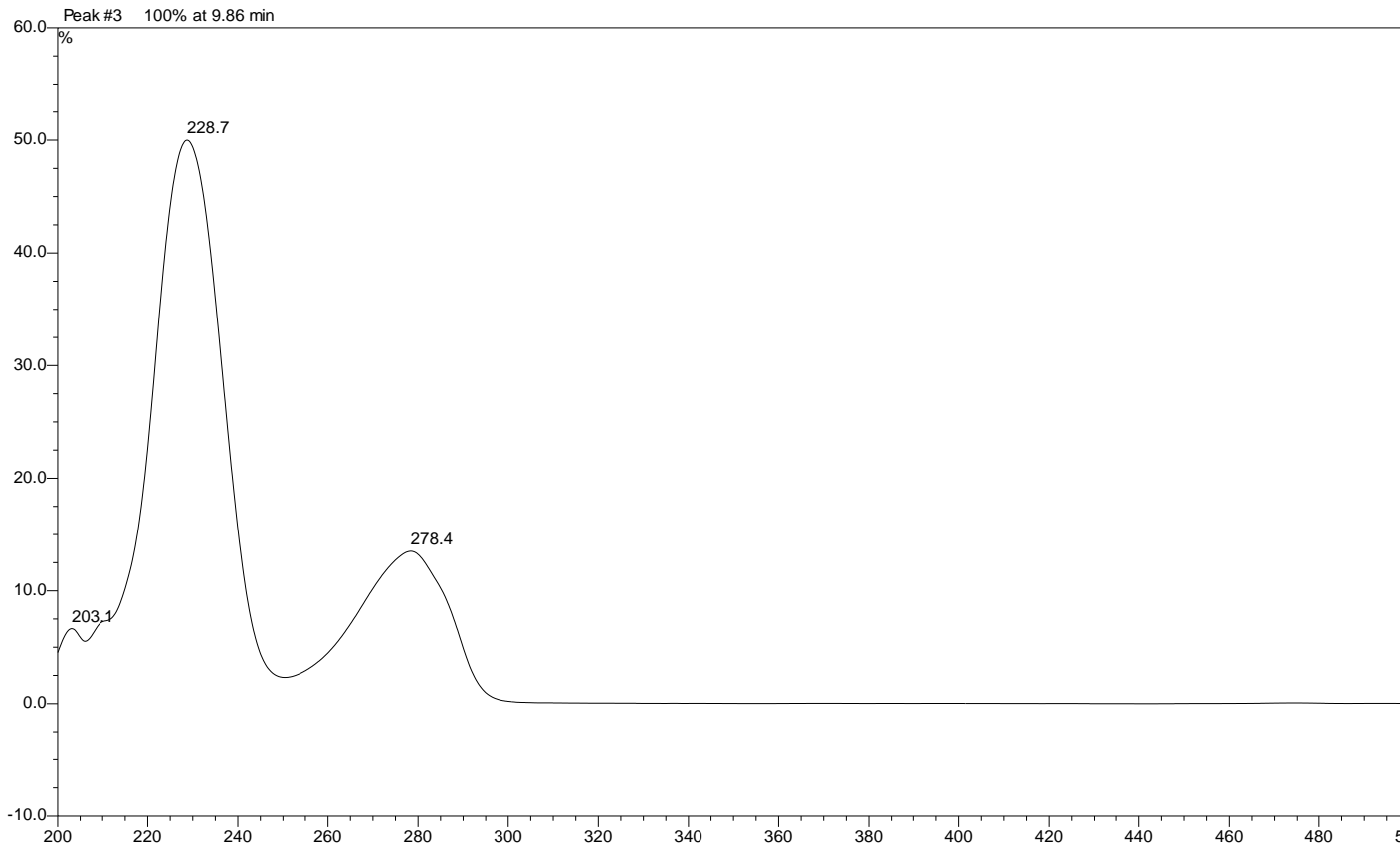
688 Fig S1

689 **Figure S1:** UV-Vis spectra of catechin, epicatechin and epigallocatechin  
690 gallate recorded from 200 to 595 nm.

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692 **Catechin**

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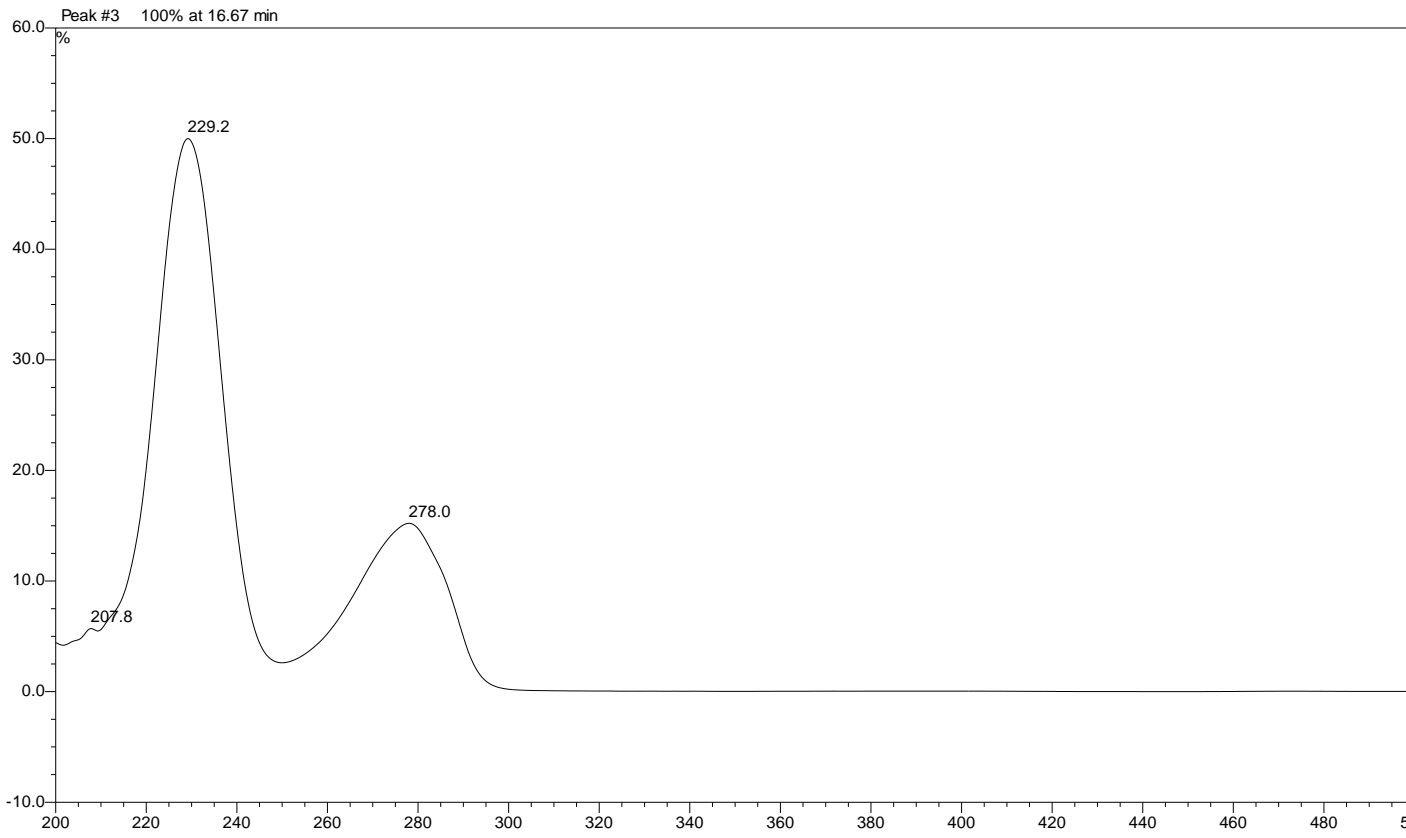
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704 **Epicatechin**

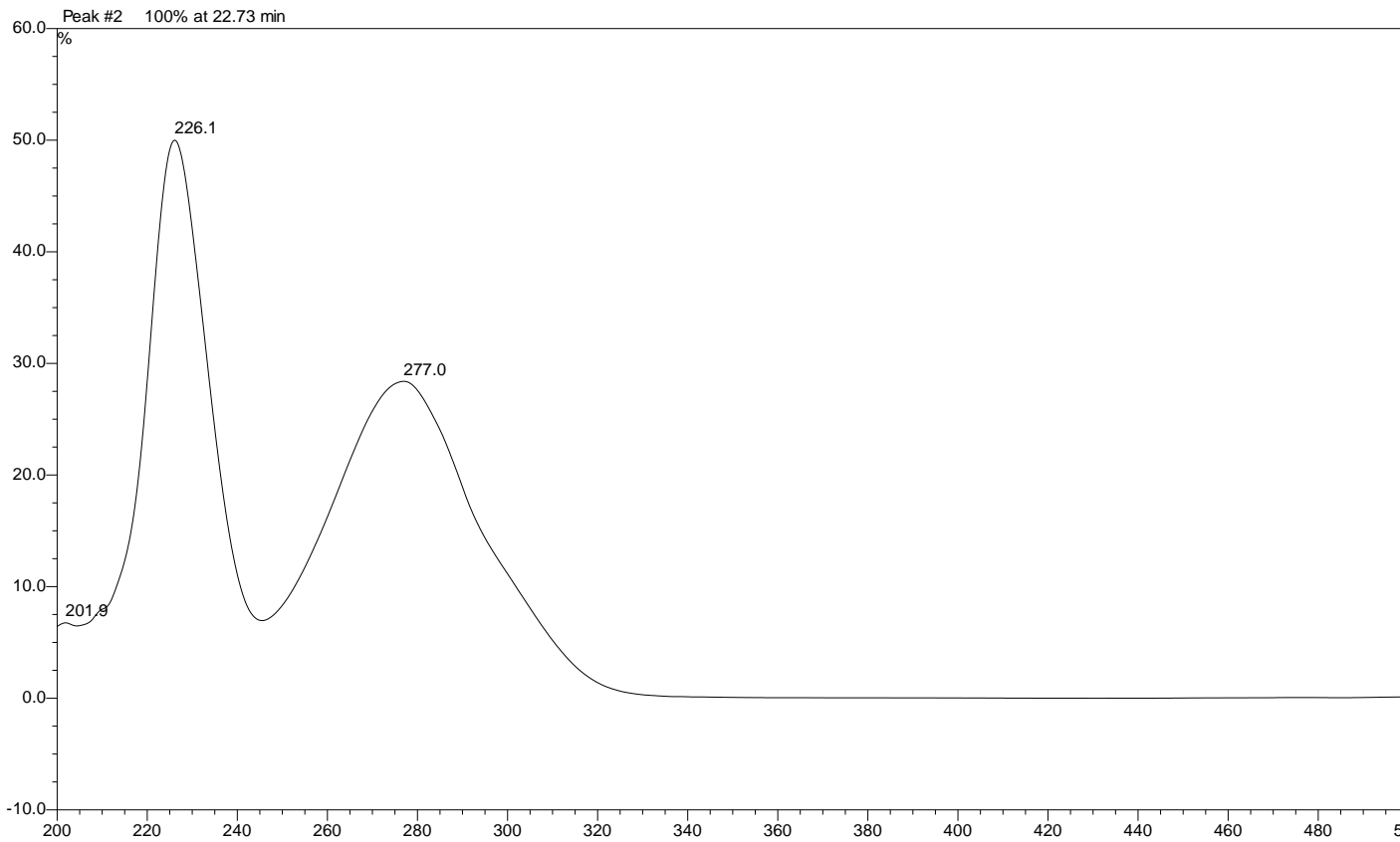
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716 **Epicatechin gallate**

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720 Supplementary **Table**

721 Fluorescence lifetimes (ns) of flavanols in the presence of different histones (Tris buffers, pH 7 and 8). Pre-exponential factors

722 are shown in brackets.

	1 mM Flavanol				2 mM Flavanol			
	pH 7		pH 8		pH 7		pH 8 <sup>b</sup>	
	$\tau_1^a$	$\tau_2$	$\tau_1$	$\tau_2$	$\tau_1$	$\tau_2$	$\tau_1$	$\tau_2$
<b>(+)-Catechin<sup>b</sup></b>								
+ Sigma histone	0.9 (75%)	1.9 (25%)	0.9 (92%)	2.5 (8%)	1.1 (82%)	1.7 (18%)	1.1 (80%)	1.9 (20%)
+ Histone sulphate	1.1 (95%)	4.0 (5%)	0.9 (75%)	2.0 (25%)	1.1 (88%)	2.1 (12%)	1.1 (88%)	2.0 (12%)
+ Roche histone	1.0 (96%)	3.5 (4%)	0.9 (80%)	2.7 (20%)	0.8 (86%)	2.5 (14%)	1.1 (93%)	3.2 (7%)
<b>(-)-Epicatechin<sup>b</sup></b>								
+ Sigma histone	0.8 (75%)	2.0 (25%)	0.9 (90%)	2.5 (10%)	1.1 (85%)	1.8 (15%)	1.0 (86%)	2.0 (14%)
+ Histone sulphate	nd <sup>c</sup>	nd	nd	nd	1.1 (87%)	1.9 (13%)	1.0 (84%)	1.9 (16%)
+ Roche histone	1.0 (92%)	2.8 (10%)	0.9 (80%)	2.3 (20%)	1.1 (90%)	3.1 (10%)	1.0 (90%)	2.7 (10%)
<b>Average</b>	0.9	2.4	0.9	2.3	1.1	2.3	1.1	2.4

723 <sup>a</sup> Experimental error is  $\pm 50$  ps; <sup>b</sup> For comparison, lifetime measurements in 0.1 M phosphate buffer at pH 8.1 gave  $\tau = 1.0$  ns for724 catechin and  $\tau_1 = 1.1$  (72%) and  $\tau_2 = 0.1$  ns (28%) for epicatechin (note: Due to the poor signal-to-noise at longer times the errors725 are significantly larger for the second lifetime ( $\tau_2$ ) and the pre-exponential factors of less than 10% may be due to a fluorescence726 contribution from impurities); <sup>c</sup> nd = not determined.