

Title: Laser induced calcium oscillations in fluorescent calcium imaging

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Abstract

Phototoxicity is the most common problem investigators may encounter when performing live cell imaging. It develops due to excess laser exposure of cells loaded with fluorophores and can lead to often overlooked but significant artifacts, such as massive increase of intracellular Ca²⁺ concentration, which would make data interpretation problematic. Because information about laser- and dye-related changes in cytoplasmic calcium concentration is very limited, we aimed to describe this phenomenon to help investigators using laser scanning confocal microscopy in a non-invasive way. Therefore, in the present study we evaluated fluorescent fluctuations, which evolved in Fluo-3/4/8 loaded mouse pancreatic acinar cells during very low intensity laser excitation. We demonstrate that after standard loading procedure (2 μM Fluo-3/4/8-AM, 30 min @ room temperature), applying 488 nm laser at as low as ca. 10 μW incident laser power (0.18 μW/μm²) at 1 Hz caused repetitive, 2-3 fold elevations of the resting intracellular fluorescence. The first latency and the pattern of the fluorescence fluctuations were laser power dependent and were related to Ca²⁺-release from intracellular stores, as they were abolished by BAPTA-AM treatment in Ca²⁺-free medium, but were not diminished by the ROS scavenger DMPO. Worryingly enough, the qualitative and quantitative features of the Ca²⁺-waves were practically indistinguishable from the responses evoked by secretagogue stimulation. Since using similar imaging conditions, a number of other cell types were reported to display spontaneous Ca²⁺ oscillations, we propose strategies to distinguish the real signals from artefacts.

Keywords: calcium imaging; Fluo-4; phototoxicity

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3 **Laser induced calcium oscillations in fluorescent calcium imaging**

4

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26

27 **Abstract.** Phototoxicity is the most common problem investigators may encounter when
28 performing live cell imaging. It develops due to excess laser exposure of cells loaded with
29 fluorophores and can lead to often overlooked but significant artifacts, such as massive
30 increase of intracellular Ca^{2+} concentration, which would make data interpretation
31 problematic. Because information about laser- and dye-related changes in cytoplasmic
32 calcium concentration is very limited, we aimed to describe this phenomenon to help
33 investigators using laser scanning confocal microscopy in a non-invasive way. Therefore, in
34 the present study we evaluated fluorescent fluctuations, which evolved in Fluo-3/4/8 loaded
35 mouse pancreatic acinar cells during very low intensity laser excitation. We demonstrate that
36 after standard loading procedure (2 μM Fluo-3/4/8-AM, 30 min at room temperature),
37 applying 488 nm laser at as low as ca. 10 μW incident laser power (0.18 $\mu\text{W}/\mu\text{m}^2$) at 1 Hz
38 caused repetitive, 2–3 fold elevations of the resting intracellular fluorescence. The first
39 latency and the pattern of the fluorescence fluctuations were laser power dependent and were
40 related to Ca^{2+} -release from intracellular stores, as they were abolished by BAPTA-AM
41 treatment in Ca^{2+} -free medium, but were not diminished by the reactive oxygen species
42 (ROS) scavenger DMPO. Worryingly enough, the qualitative and quantitative features of the
43 Ca^{2+} -waves were practically indistinguishable from the responses evoked by secretagogue
44 stimulation. Since using similar imaging conditions, a number of other cell types were
45 reported to display spontaneous Ca^{2+} oscillations, we propose strategies to distinguish the real
46 signals from artifacts.

47
48 **Abbreviations:** $[\text{Ca}^{2+}]_i$, intracellular calcium concentration; BSA, bovine serum albumin; cch,
49 carbachol; CICR, calcium induced calcium release; ER, endoplasmic reticulum; IP_3R , inositol
50 trisphosphate receptor; ROI, region of interest; ROS, reactive oxygen species; RyR, ryanodine
51 receptor; SERCA, sarco-endoplasmic reticulum calcium ATP-ase; SOCE, store operated
52 calcium entry.

53

54

55 **Introduction**

56

57 Ca^{2+} is an important second messenger in the cell, which controls many cellular functions
58 such as muscle contraction, exocytosis, gene expression, proliferation and cell death. In order
59 to fulfill its mission, it is essential to maintain intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) low at
60 rest, but to allow it rapidly and transiently rise during excitation. For example, in pancreatic

61 acinar cells $[Ca^{2+}]_i$ is elevated by Ca^{2+} -release from the endoplasmic reticulum (ER) through
62 inositol trisphosphate receptors (IP_3R) and ryanodine receptors (RyR) upon secretagogue
63 stimulation to trigger exocytosis of zymogen containing vesicles (Straub et al. 2000, Petersen
64 et al. 2007, Leite et al. 2002, Williams et al. 1978, Habara et al. 1994). Sustained stimulation
65 leads to ER depletion and the activation of store operated calcium entry (SOCE) to support
66 prolonged Ca^{2+} signals and ER reload (Lewis et al. 2007, Smyth et al. 2010, Putney et al.
67 2007). Afterwards, $[Ca^{2+}]_i$ is restored by the sarco-endoplasmic reticulum Ca^{2+} -ATP-ase
68 (SERCA) and the plasma membrane Ca^{2+} pump (PMCA). They are also responsible for
69 keeping $[Ca^{2+}]_i$ stable and low (ca. 100 nM) in unstimulated cells (Yule 2010).

70 Certainly, biomedical researchers are particularly interested in measuring the changes
71 of $[Ca^{2+}]_i$ because of its critical influence on the cell's fate. Their scientific ambition is
72 supported by the development of fluorescent Ca^{2+} imaging techniques in the past few decades.
73 The simplest and most popular Ca^{2+} imaging tools are the Ca^{2+} indicator fluorescent dyes
74 from the Fluo family (Fluo-3/4/8) (Minta et al. 1989, Gee et al. 2000). These dyes are also
75 available in acetoxymethylester (AM)-conjugated form which easily cross the plasma
76 membrane, but inactive (does not bind Ca^{2+}). The dye attains activity after the AM group is
77 enzymatically hydrolyzed by intracellular esterases, which also makes the resulting dye water
78 soluble to prevent the dye escaping from the cell.

79 An important problem of fluorescent imaging is that exposure of the fluorophore to
80 high intensity focused light is required for excitation and subsequent fluorescent emission;
81 however, the illuminating light itself is the source of two undesirable consequences:
82 phototoxicity and photobleaching (Pawley et al. 2006, Hoebe et al. 2007, Rohrbach et al.
83 2005, Collins et al. 2014, Bootman et al. 2013). Photobleaching (fading) is mainly due to
84 classic photodestruction, whereas phototoxicity is due to the photochemical reaction of the
85 excited fluorophore with molecular oxygen, which produces reactive oxygen species (ROS).
86 ROS oxidize cellular components that results in cell damage (phototoxicity), and also react
87 with the fluorophore, which contributes to fluorescent signal loss (photobleaching) (Pawley et
88 al. 2006, Hoebe et al. 2007, Rohrbach et al. 2005, Collins et al. 2014, Bootman et al. 2013).
89 The major complication of phototoxicity during live cell imaging is not the reduced cell
90 viability itself, but the unusual behavior of the damaged cell, which can contaminate the
91 detected signal and deceive the investigator (Pawley et al. 2006, Hoebe et al. 2007, Rohrbach
92 et al. 2005, Collins et al. 2014, Bootman et al. 2013).

93 An example of such an artifact is light induced Ca^{2+} elevation in cells loaded with
94 Fluo- calcium sensitive dyes. While this issue could affect most of the confocal microscopy

95 users who perform Ca^{2+} imaging, its literature is limited to only a couple of papers
96 ([McDonald et al. 2012](#), [Knight et al. 2003](#)). These reports describe light-induced Ca^{2+}
97 transients in Fluo-3 AM-loaded smooth muscle cells and in Fluo-4 AM-loaded cultured
98 chondrocytes during epifluorescent imaging using light emitting diodes and during laser-
99 scanning confocal microscopy, respectively. In the present paper repetitive, laser activated
100 Ca^{2+} -release events were evaluated in Fluo-loaded pancreatic acinar cells and other cell types
101 using laser scanning confocal microscopy to help investigators identify light-related artifacts.
102 Moreover, strategies to overcome the problem are also offered.

103

104 **Materials and Methods**

105

106 *Chemicals*

107 Fluo-3/4/8-AM and Fura-Red-AM was purchased from Molecular Probes (ThermoFisher
108 Scientific). All other materials were purchased from Sigma, unless otherwise specified.

109

110 *Pancreatic acinar cell isolation*

111 Experiments were performed in accordance with EU (86/609/EEC) guideline under a license
112 obtained from the Scientific Committee on Animal Health and Welfare of the University of
113 Debrecen. Pancreatic acinar cells were freshly isolated from mouse pancreas as described
114 previously. Briefly, 2–4 months old NMRI mice of both genders were euthanized by cervical
115 dislocation and the pancreas was rapidly removed. The tissue was injected with F12/DMEM
116 medium containing 100 U/ml collagenase P (Roche), 0.1 mg/ml trypsin inhibitor and 2.5
117 mg/ml BSA and then incubated in this solution for 30 minutes in a 37°C shaking water bath.
118 The media were continuously gassed with carbogen. The tissue was dissociated by pipetting
119 4–6 times using a 5 ml serological pipette. The cell clumps then were filtered through mesh
120 #60 (150 μm). The filtrate was layered on the top of 400 mg/ml BSA and washed through the
121 medium by gentle centrifugation. The cell pellet was resuspended and collected by
122 centrifugation. Acinar cell clumps were gently resuspended in F12/DMEM medium and kept
123 gassed at room temperature until use ([Geyer et al. 2015](#)).

124

125 *Cell cultures*

126 HEK293 cells and HaCaT keratinocytes were cultured in DMEM medium supplemented with
127 10% fetal bovine serum (FBS) at 37° in a CO_2 thermostat ([Geyer et al. 2015](#)). Cells were
128 allowed to grow to 60–70% confluence.

129 *Intracellular Ca²⁺ imaging*

130 Acinar cell clumps and other cell cultures were loaded with 0.5–2 μ M Fluo-4-AM Ca²⁺-
131 sensitive dye for 30 minutes at room temperature (exact concentrations used are indicated in
132 the text). Cells were plated on glass coverslips and mounted on a perfusion chamber. After
133 perfusion with Tyrode's solution containing (in mM): 140 NaCl, 5 KCl, 2 MgCl₂ and 10
134 HEPES, pH = 7.2 with or without 1.8 CaCl₂, fluorescence was monitored in time series
135 measurements using a Zeiss LSM 5 LIVE confocal microscope equipped with a 40 \times oil
136 immersion objective for most experiments or a Zeiss LSM 510 META confocal microscope
137 with a similar objective for some experiments. Fluo-4 was excited at 488 nm and the emitted
138 light was collected through a 500–525 nm band-pass filter. The pinhole was set to correspond
139 to ca. 5 μ m tissue section widths (Geyer et al. 2015). In some experiments Fluo-4-AM was
140 co-loaded with Fura-Red AM (2 and 6 μ M respectively). In these experiments, both
141 fluorophores were excited with the 488 nm argon laser, the emitted light was divided by a 635
142 nm beamsplitter and detected simultaneously after filtered with a 500–525 nm bandpass filter
143 for the green channel or no filter for the red channel. In some experiments, cells were treated
144 with 20 μ M BAPTA AM for 20 minutes or 2 mM tetracaine (tetracaine was also included in
145 the perfusion solution). To test the role of ROS, 500 μ M 5,5-dimethyl-pyrroline N-oxide
146 (DMPO) was included into the bath solution (pH = 7.2). Fluorescence emission data of single
147 cells was analyzed and F/F₀ ratio was calculated after background subtraction using Zeiss
148 ZEN 2009 and Microsoft Excel software, respectively. Spatio-temporal analysis of Ca²⁺
149 waves was performed using high frequency line-scan imaging (500 lines/s).

150

151 *Statistics*

152 Averages are expressed as mean \pm SEM (standard error of the mean). Statistical analysis was
153 performed using Student's t-test. Threshold for statistically significant differences as
154 compared to the respective control was set at * $p < 0.01$.

155

156 **Results**

157

158 *Spontaneous Ca²⁺ oscillations observed in x-y imaging mode*

159 The data presented here were obtained in enzymatically isolated mouse pancreatic acinar cell
160 clumps of various sizes (ca. 10–30 cells) using a Zeiss LSM 5 LIVE line-scanning confocal
161 microscope. The cells showed retained polarized morphology, characterized by apical
162 granules. The cell clumps maintained typical acinar architecture. No obvious signs of cell

163 damage (e.g. blebbing) were observed either before or after the experiments (Figure 1A).
164 Importantly, we intended to avoid phototoxicity by optimizing the dye loading conditions so
165 the resting fluorescence fell above the lowest measurable intensity. Using 1 mW laser power
166 output, the resting intracellular fluorescence (872 ± 72 arbitrary unit, AU) was only 3 fold
167 higher than the background fluorescence (288 ± 14 AU). Notably, this laser intensity
168 corresponds to only 1% of the maximum power output of our argon laser, which is a typical
169 setting for confocal imaging of live cells. In this case, due to various losses in the imaging
170 system, 10 μ W laser power is transmitted through the objective and the power density of the
171 light is $0.18 \mu\text{W}/\mu\text{m}^2$ and the dwell time is 972 μs .

172 Acinar cells were loaded with 2 μM Fluo-4-AM for 30 minutes and mounted on
173 coverslips of a perfusion chamber. Cells were washed with physiological saline solution and
174 were excited repetitively with a 488 nm laser beam at 1 mW, 1 Hz using the x-y scan mode.
175 Because unstimulated acinar cells should exhibit stable basal $[\text{Ca}^{2+}]_i$ (Petersen et al. 2007), we
176 were surprised to observe robust, repetitive fluctuations of intracellular fluorescence in most
177 of the cells, which appeared within 3 minutes after the first frame and rapidly expanded in the
178 whole cell. Fluorescence seemed to increase in most cells of the specimen. An example of
179 such a fluorescence oscillation is shown in Figure 1B.

180 Although, a number of cell types (Wang et al. 2006, Vukcevic et al. 2010, Fedoryak et
181 al. 2004, Johnston et al. 2005) were shown to display physiologically relevant spontaneous
182 Ca^{2+} oscillations, resting oscillatory behavior is not the intrinsic property of acinar cells,
183 which suggest that what we have seen was a light-induced artifact. One would expect light-
184 induced artifacts to be dependent on exciting light intensity and other imaging conditions. In
185 contrast, if our oscillations were due to spontaneous, intrinsic biological activity of the cell, it
186 wouldn't be laser power dependent. Therefore, we aimed to find out how this phenomenon
187 could have triggered and to test its light dose-response relationship.

188 The oscillations apparently had a dye-related origin, as they had earlier onset and higher
189 amplitude at higher dye concentrations and could be completely prevented by using lower
190 extracellular Fluo-4-AM concentrations. Also, our experiments using different laser powers
191 demonstrated that the qualitative features of the fluorescence highly depended on the laser
192 exposure. In comparison with 1 mW, at 3 mW (ca. 30 μW incident light, $0.54 \mu\text{W}/\mu\text{m}^2$ power
193 density) we detected long-lasting elevations of the fluorescence with depressed oscillatory
194 behavior and earlier onset, but similar spiking frequency ($0.99 \pm 0.03/\text{min}$ vs. 0.95
195 $\pm 0.08/\text{min}$, Figure 1B–F). The first peak latency of fluorescence was 237 ± 6 sec for 1 mW
196 and 140 ± 13 s for 3 mW (Figure 1E). Although, most of the cells responded to both laser

197 intensities, the ratio of active cells varied between specimens, with >90% in some cases.
198 However, when cells were imaged using lower laser power (0.5 mW laser output = 5 μ W
199 incident light power and 0.09 μ W/ μ m² power density) at 0.5 Hz, fluorescence was stable
200 during the 12 minutes recording time except for the insignificant, but continuous reduction of
201 basal fluorescence, because of photobleaching (Figure 1D). Very similar oscillatory behavior
202 was demonstrated in Fluo-3 and Fluo-8 loaded pancreatic acinar cells, too (supplementary
203 Figure S1.).

204 Our biggest concern about the phenomenon was that the light-activated oscillatory behavior
205 was practically indistinguishable from the activity elicited by the parasympathic
206 neurotransmitter acetylcholin-analogue carbachol (cch), which we often use to test pancreatic
207 acinar cell function. This is demonstrated in Figure 1G, which shows a typical response of
208 acinar cells to 100 and 200 nM cch under “non-invasive” imaging conditions (i.e. 0.5 mW
209 laser power, 0.5 Hz). These original records clearly show that the signal amplitude and the
210 oscillation frequency were very similar for the cch and the laser-induced signals.

211 To exclude the possibility that the oscillations can be only elicited by our Zeiss LSM 5
212 LIVE line-scanning high-speed confocal microscope, similar experiments were performed
213 using a Zeiss LSM 510 META microscope. Similar spontaneous repetitive fluorescence
214 spikes could have been also observed (supplementary Figure S2.).

215

216 *Ca²⁺ oscillations in HEK293 cells and HaCaT keratinocytes*

217 In order to determine whether this artefact is restricted to pancreatic acinar cells or occurs in
218 other cell types too, we performed similar experiments using Fluo-4-AM loaded HEK293
219 cells and HaCaT keratinocytes. Both cell types showed similar spontaneous transient
220 elevation of [Ca²⁺]_i (Figure 2A and B). Interestingly, no transients could be triggered again on
221 the same cells using the same imaging conditions.

222

223 *Detailed investigation and prevention of spontaneous Ca²⁺ oscillations in pancreatic acinar* 224 *cells*

225 Next, in order to prevent the spontaneous calcium oscillations, we aimed to learn more about
226 them. In previous studies, ROS production was shown to be responsible for phototoxicity
227 (Bootman et al. 2013, Knight et al. 2003, Dixit and Richard 2003, Grzelak et al, 2001);
228 therefore, we tested the role of ROS in laser-induced fluorescent oscillations by using the
229 ROS scavenger DMPO. Acinar cells were treated with the reagent for 10 minutes before the
230 experiment and cells were continuously perfused with physiological saline solution

231 supplemented with the reducing agent during imaging. Surprisingly, the treatment did not
232 suppress the fluorescent fluctuations, which implies that ROS is not required to generate the
233 oscillatory signal (Figure 3A). This result argues against the hypothesis that ROS mediates
234 fluorescent fluctuations in Fluo-4 loaded cells (Knight et al. 2003).

235 To clarify whether the laser-induced fluorescence change was really due to $[Ca^{2+}]_i$
236 fluctuations, another Ca^{2+} indicator was also used for signal detection. For this purpose Fura-
237 Red was chosen because it allows simultaneous Fluo-4 recordings (see Materials and methods
238 for details) but has very different photochemical properties than Fluo-4. Fura-Red is a Ca^{2+}
239 quenching fluorophore, which means that the increase in $[Ca^{2+}]_i$ is reported by a decrease in
240 its emission when excited at 488 nm (Thomas et al. 2000). Moreover, it is less susceptible to
241 photobleaching and Fura-Red loaded cells show weaker phototoxicity (Rohrbach et al. 2005).
242 Because of these very different optical and chemical characteristics, we assumed that if the
243 oscillations observed in the Fluo-4 signal were not due to changes in the intracellular Ca^{2+}
244 concentration, Fura-Red should not have reported the change either (Lipp and Niggli 1993).
245 Consequently, if typical Fura-Red responses during laser excitation were detected, it would
246 rather be attributable to changes in $[Ca^{2+}]_i$. To test this, cells were co-loaded with Fluo-4 and
247 Fura-Red and the emissions were detected simultaneously (Lipp and Niggli 1993). A
248 representative record of such an experiment is displayed in Figure 3B, showing that the
249 increase in Fluo-4 fluorescence was tightly associated with the decrease in Fura-Red
250 emission.

251 More importantly, loading cells with the cell permeable Ca^{2+} chelator BAPTA-AM
252 prevented the fluorescent events (Figure 3C). These results imply that the observed
253 fluorescence fluctuation was due to repetitive changes of $[Ca^{2+}]_i$.

254 Next, experiments were designed to identify the source of Ca^{2+} . Exchanging the bath
255 to Ca^{2+} -free saline neither influenced the amplitude, nor the shape of the spikes, whereas
256 spiking was abolished by 2 mM tetracaine (Figure 3D and E), which inhibits Ca^{2+} -release
257 channels in this concentration. These results strongly suggest that Ca^{2+} was released from the
258 ER.

259

260 *Ca²⁺ oscillations on line scan images*

261 Because the spatio-temporal characteristics of Ca^{2+} signals are often visualized in line-scan
262 mode, we investigated whether Ca^{2+} -release can be provoked in this mode too, when a single
263 pixel line is excited repetitively at the rate of 500 times per second, at laser intensities ranging
264 from 0.2 mW to 3 mW (2–30 μ W). In Figure 4A–C, representative time series line-scan

265 recordings and plots are shown. The scanning line was set across the cell in the apico-basal
266 direction (Figure 4A, B, C, left side, yellow lines). The frames used for the selection of the
267 line were always taken using 0.2 mW laser power output to prevent spontaneous Ca^{2+} release
268 during the line selection process. Brightness and contrast of the “line select” images were
269 improved after measurement to allow better visibility.

270 When the line was excited using 3 mW laser power (Figure 4A), a robust apico-basal
271 Ca^{2+} wave developed immediately (Figure 4, black line), which was followed by a second
272 one. Importantly, cell morphology only changed (i.e. blebbing developed) 75 s (37500 line
273 scans) after imaging started (Figure 4A, white arrowhead), much later than the Ca^{2+} -
274 oscillations appeared. In order to prevent obvious signs of phototoxicity and to find the lowest
275 laser power required to elicit the oscillations, laser power was gradually decreased (Figure
276 4B, C). Although, excitation using 0.2 mW laser power setting did not cause measurable
277 photobleaching or cell damage, it still elicited significant Ca^{2+} release (Figure 4C). It has to be
278 mentioned that the 0.2 mW setting is the lowest possible laser emission setting and the 500
279 FPS is the lowest possible line scanning rate for the model of microscope used and both fell
280 below the typically used settings.

281 Line scan recordings at high temporal resolution of the apical region of the cells (Figure 5),
282 revealed proportional relationship between the laser power output and the onset of the Ca^{2+}
283 signal.

284

285 Discussion

286

287 Overall, we report an uncommon, but significant methodological problem that exciting laser
288 radiation after conventional Fluo-4-AM loading protocol and microscope settings causes
289 intracellular Ca^{2+} release. Whilst earlier studies have shown that high illumination levels or
290 sustained illumination can lead to various cytotoxic effects (including Ca^{2+} transients) (Smyth
291 et al. 2010, Putney 2007), we have demonstrated that in case of our cells even very low levels
292 of excitation and dye-concentration may cause calcium oscillations. We wish to highlight that
293 these setting are well below the range normally considered safe for imaging (10–30 μW).
294 Notably, Knight et al. showed that 488 nm laser of similar power (15–30 μW) induced Ca^{2+} -
295 oscillations in chondrocytes (Knight et al. 2003).

296 Based on our current data we propose that laser exposure of Fluo-4 or other dyes in the Fluo-
297 family produces an unknown derivative, which causes Ca^{2+} -release from the ER by activating
298 IP_3Rs . We found the that the changes in Fluo-4 fluorescence were due to changes in

299 intracellular Ca^{2+} levels and the source of Ca^{2+} was shown to be intracellular, because
300 removing extracellular Ca^{2+} did not suppress the Ca^{2+} waves. We have also shown that the
301 amplitude and calcium release kinetics of the laser-induced oscillations in mouse pancreatic
302 acinar cells are comparable to those triggered by 100 or 200 nM carbachol. The only major
303 intracellular compartment capable of such Ca^{2+} release is the ER. Although, RyRs are also
304 involved in the Ca^{2+} release process in pancreatic acinar cells, we tend to blame IP_3Rs as a
305 culprit to initiate the oscillations, because skeletal muscle fibers that are poor in IP_3Rs but
306 very rich in RyRs, (Fill and Copello 2002) do not show similar laser-induced events
307 (Csernoch 2007). In addition, HEK293 cells were reported to lack endogenous RyR channels,
308 but express IP_3Rs (Tong et al. 1999, Alzayady et al. 2016).

309 Ca^{2+} -wave expansion in our cells requires the dynamic cooperation of both, unevenly
310 distributed, but connected parts of the main intracellular Ca^{2+} compartment, the ER. IP_3Rs are
311 primarily located in the apical ER, whereas RyRs can be found throughout the ER, but most
312 abundantly in the supranuclear-basal region. Therefore, physiological secretagogue
313 stimulation causes Ca^{2+} waves that are always initiated by IP_3R activation on the apical side
314 of the ER and propagate towards the basal end *via* CICR (Petersen 2005, Petersen and
315 Tepikin 2008, Petersen 2014). The intracellular Ca^{2+} dynamics of laser-induced oscillations
316 are very similar to those of secretagogue-induced responses, further suggesting the major role
317 of IP_3Rs in the process. The apico-basal propagation of calcium signal suggests the
318 involvement of IP_3Rs in the initiation of the light-activated calcium signal. In Figure 5, the
319 stepwise increase of Ca^{2+} -level on the apical side of cells triggered by 0.2 and 1 mW laser
320 power suggests that the waves might be formed by a multi-step process. This can be explained
321 by the sequential opening of Ca^{2+} channels or the exhaustion of Ca^{2+} -buffering capacity of the
322 apical portion of the cell.

323 The current study initially investigated the mechanism of light induced artifacts in mouse
324 pancreatic acinar cells, but later revealed that the problem is not limited to this cell type and
325 looks to be a general phenomenon.

326 It should be highlighted that decreasing Fluo-loading may not offer an adequate
327 strategy to avoid laser-induced Ca^{2+} release in all cells because it compromises the signal to
328 noise ratio (the resting fluorescence at 1 mW was already only 3 times higher than the
329 background). Instead, we suggest finding the balance by minimizing both the level of dye
330 loading and the cumulative incident light intensity by using low imaging rate with the lowest
331 light intensity and dwell time possible. It must also be noted that in case of line-scan imaging

332 we could not prevent the formation of light-induced Ca^{2+} release even at the lowest possible
333 excitation level and lowest dye-loading.

334 In conclusion, during laser-scanning microscopy possible artifacts due to laser
335 excitation should be taken into account, even when low power settings are used and in some
336 cases laser scanning methods may not be useable for calcium imaging.

337

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344

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463

464 **Figure legends**

465 **Figure 1.** Features of laser induced changes of Fluo-4 fluorescence in pancreatic acinar cells.
466 **A.** Bright-field microscopy image of a pancreatic acinar cell clump. **B. C. D.** Representative
467 time series fluorescent records of Fluo-4 loaded single acinar cells using 1, 3 and 0.5 mW
468 laser power settings, respectively. **E.** Laser power dependence of the latency of the first

469 fluorescence peak (* $p < 0.01$). **F.** Laser power dependence of the frequency of fluorescent
470 oscillation. **G.** Carbachol (cch)-induced Ca^{2+} oscillations in single pancreatic acinar cells.
471

472 **Figure 2.** Laser induced fluorescent oscillations in HEK293 cells and HaCaT keratinocytes.

473 **A. B.** Fluorescent images of Fluo-4 loaded (2 μM , 30 min) HEK293 cells and HaCaT
474 keratinocytes are shown before imaging (top). Representative fluorescent records using 1%
475 (for HaCaT) and 3% (for HEK293) laser settings. Different curves represent examples of
476 fluorescence of different cells.

477

478 **Figure 3.** The laser induced fluorescent oscillation is due to Ca^{2+} -release from the ER.

479 Control representative curves are shown with dashed lines and treatments (of different cells)
480 are shown with solid lines. Each line is a representative fluorescence intensity curve of a cell
481 from a run. 3 runs (treatments) were performed for each condition on different groups of cells.

482 **A.** Fluorescent emissions recorded in pancreatic acinar cells under control conditions and in
483 the presence of the ROS scavenger DMPO. **B.** Fluorescent oscillations recorded in a cell,
484 which was co-loaded with Fluo-4-AM and Fura-Red-AM. **C.** The oscillations are abolished
485 by BAPTA-AM treatment. **D.** Replacing Ca^{2+} containing extracellular solution to Ca^{2+} -free
486 medium did not influence the oscillatory pattern. **E.** Tetracaine diminished the laser-induced
487 Ca^{2+} -release.

488

489 **Figure 4.** Spatio-temporal characteristics of laser-induced Ca^{2+} -waves in line scan mode. **A.**

490 **B. C.** Line scan representative records and plots of cells using 3, 1 and 0.2 mW laser outputs,
491 respectively. The scanning line was placed across the cells in the apico-basal direction, as
492 indicated by the yellow lines. The apical regions of the cells are shown by black, whereas the
493 red arrowheads show the basal regions. The fluorescence changes of these regions are shown
494 by black and red curves, respectively.

495

496 **Figure 5.** High-resolution plot of the initial response to various levels of laser excitation in
497 line scan mode. The first 20 seconds of apical line scan representative records of Figure 4,
498 where laser power outputs of 3 mW (black with white centerline), 1 mW (dark grey) and 0.2
499 (light grey) mW were used.

500

501 **Figure S1.** Laser emission-evoked fluorescent oscillations in Fluo-4 AM-loaded pancreatic
502 acinar cells.

503

504 **Figure S2.** Laser emission-evoked fluorescent oscillations in Fluo-3 and 8 AM-loaded

505 pancreatic acinar cells.

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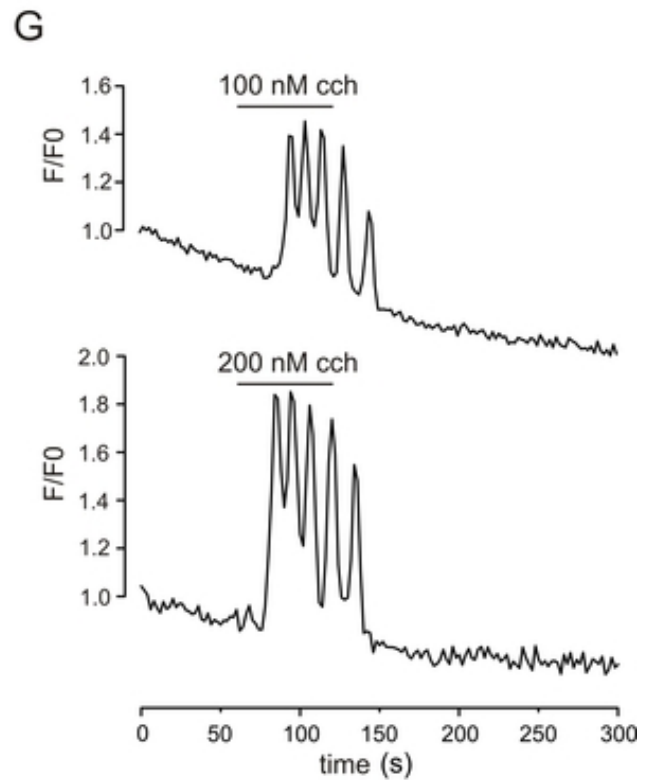
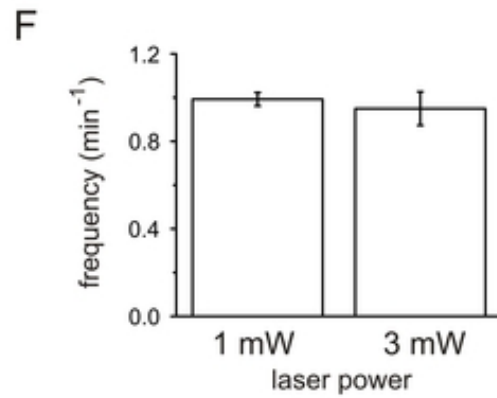
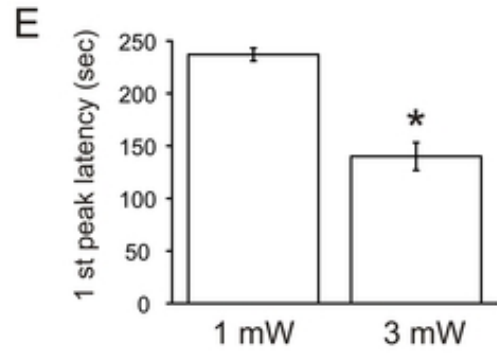
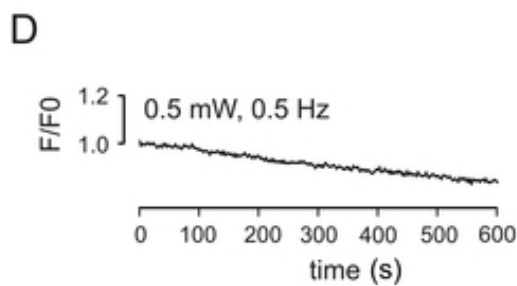
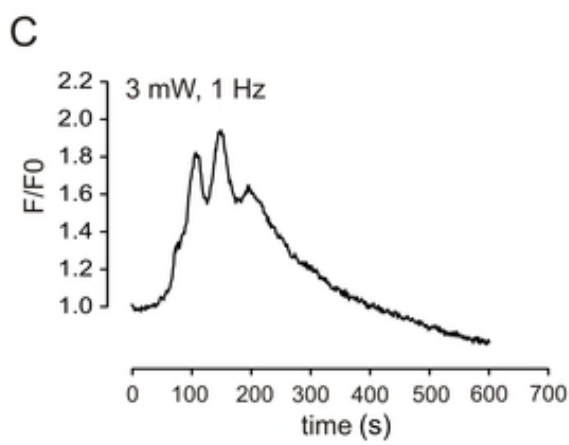
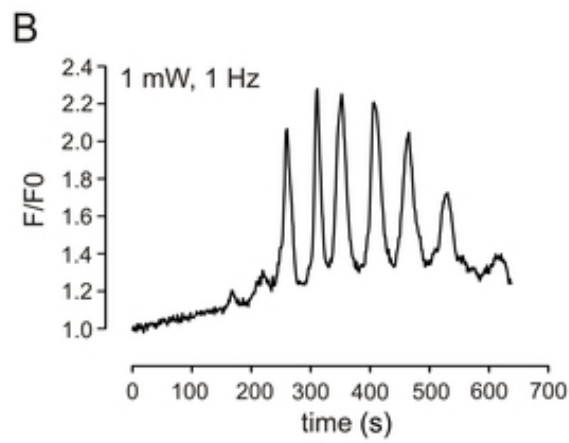
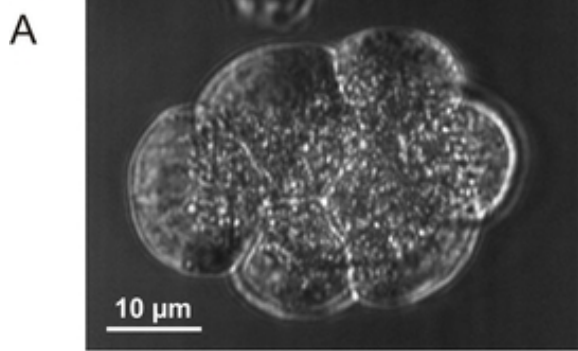
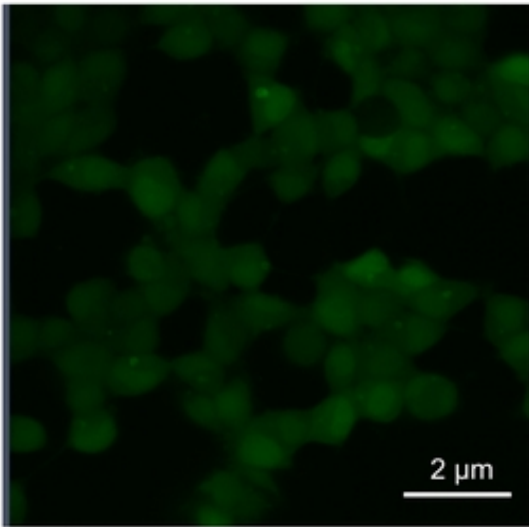


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A

HEK 293



B

HACAT

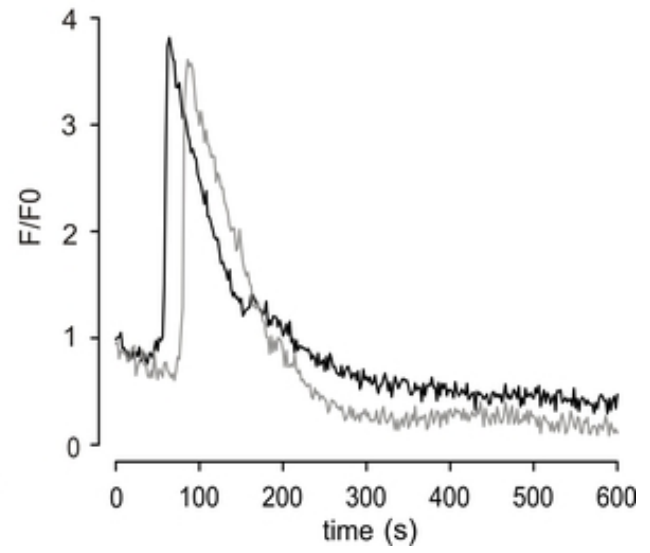
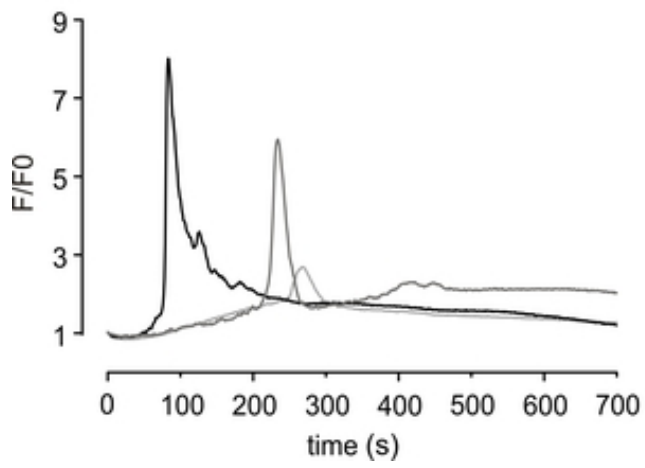
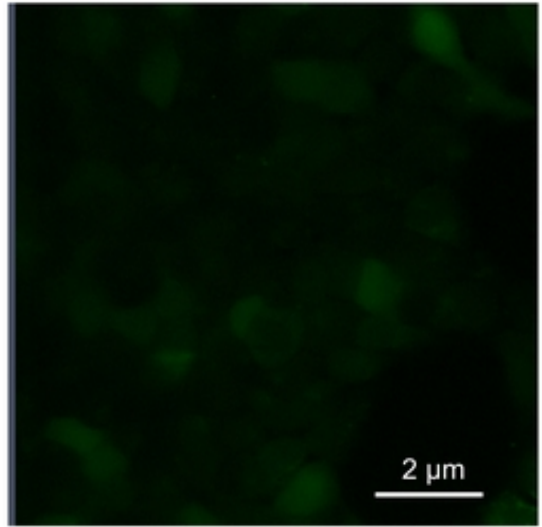


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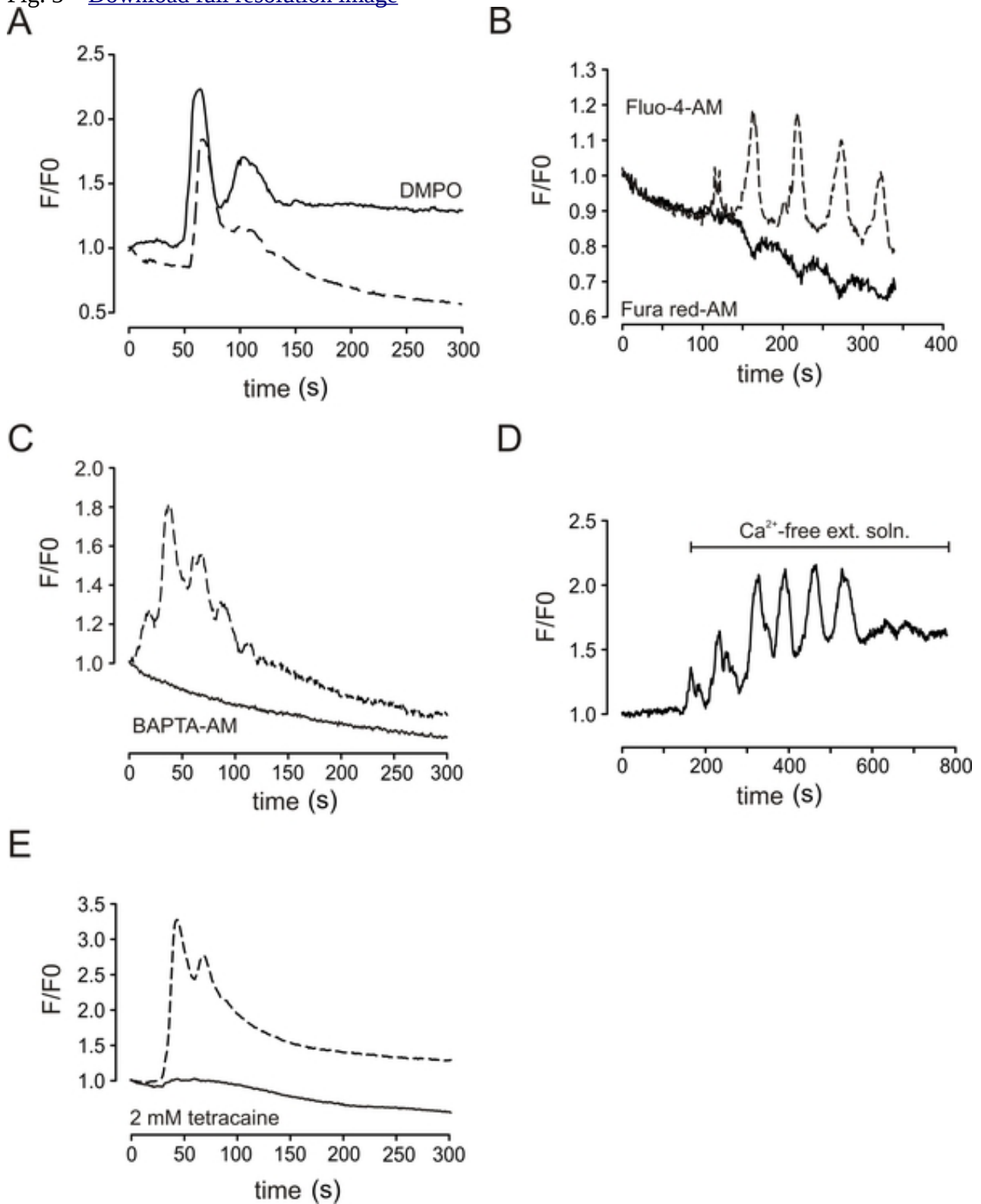


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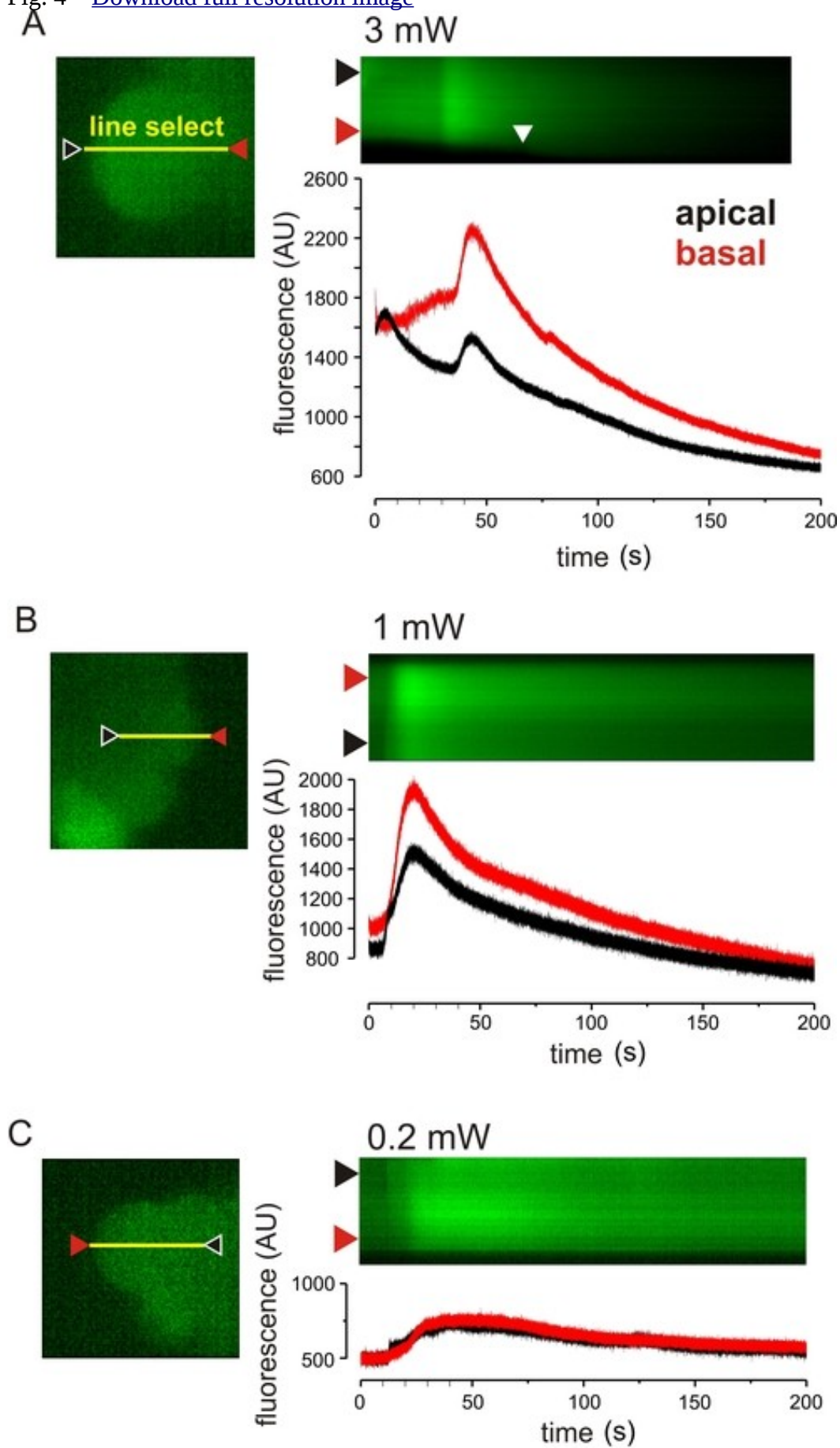


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