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1 Automated screening of AURKA activity based on a genetically 2 encoded FRET biosensor using Fluorescence Lifetime Imaging 3 Microscopy

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11

12 **Abstract**

13 Fluorescence Lifetime Imaging Microscopy (FLIM) is a robust tool to measure Förster
14 Resonance Energy Transfer (FRET) between two fluorescent proteins, mainly when using
15 genetically-encoded FRET biosensors. It is then possible to monitor biological processes such
16 as kinase activity with a good spatiotemporal resolution and accuracy. Therefore, it is of interest
17 to improve this methodology for future high content screening purposes. We here implement a
18 time-gated FLIM microscope that can image and quantify fluorescence lifetime with a higher
19 speed than conventional techniques such as Time-Correlated Single Photon Counting (TCSPC).
20 We then improve our system to perform automatic screen analysis in a 96-well plate format.
21 Moreover, we use a FRET biosensor of AURKA activity, a mitotic kinase involved in several
22 epithelial cancers. Our results show that our system is suitable to measure FRET within our
23 biosensor paving the way to the screening of novel compounds, potentially allowing to find
24 new inhibitors of AURKA activity.

25 **Keywords:** fluorescence lifetime, FRET biosensor, screening, AURKA

26 **Introduction**

27 Since several years, FLIM has been widely used to measure the fluorescence lifetime of
28 fluorescent proteins (Becker, 2012). It is an intrinsic parameter independent of concentration,
29 which, however, depends on the environment of the fluorophore such as FRET, a non-radiative
30 energy transfer from excited fluorescent donor dipole to a close acceptor dipole (<10nm). Given
31 that, a decrease of the donor fluorescence lifetime is observed when FRET is present (Lakowicz,
32 2006) and lifetime is often used as a way to measure FRET. In this light, FRET-FLIM has been

33 widely used to investigate protein-protein interactions (Kenworthy, 2001; Padilla-Parra and
34 Tramier, 2012), cleavage activity of proteases (Horton et al., 2007), protein oligomerization
35 (Richert et al., 2015). Originally developed for calcium signalling (Miyawaki et al., 1997),
36 genetically encoded-FRET biosensors have opened new perspectives in quantitative
37 fluorescence microscopy. These biosensors are now powerful tools to investigate biological
38 events in living cells (Hochreiter et al., 2015). In addition, the development of kinase FRET
39 biosensors allowed to track the activity of kinases within living cells in time and space (Sizaire
40 and Tramier, 2017; Zhang et al., 2001). High-content screening (HCS) is a widespread
41 methodology to screen and design new inhibitors and it can be adapted to microscopy setups
42 (Thomas, 2010). Different systems combining screening with FRET-FLIM have arisen
43 allowing to study protein-protein interaction (Guzmán et al., 2016; Margineanu et al., 2016) or
44 to find new epigenetics markers in cancer (Liu et al., 2019). More recently, screening
45 combination with FRET-FLIM seems to be a new promising methodology for pharmacological
46 screenings (Guo et al., 2019). In this article, we present a novel pipeline, consisting of an
47 automated FRET-FLIM microscope used to screen AURKA activity with a FRET biosensor.
48 AURKA is a serine/threonine kinase and has been first described as a mitotic kinase in
49 *Drosophila* (Glover et al., 1995). It plays several roles in cell division such as mitotic entry,
50 maturation of centrosomes, assembly of the mitotic spindle and the midbody (Nikonova et al.,
51 2013). AURKA is known to be an oncogene and is overexpressed in several epithelial cancers
52 (Bischoff et al., 1998; Sen et al., 1997). Our team has developed a genetically encoded FRET
53 biosensor of AURKA which consists of the whole kinase flanked by the GFP and mCherry
54 fluorophores (Bertolin et al., 2016). We showed that the conformational change of the kinase,
55 which is indicative of its activation, modifies the proximity between GFP and mCherry
56 increasing FRET efficiency. This biosensor has been validated to be a suitable tool to find new
57 non-mitotic roles of the kinase, particularly at mitochondria to control organelle dynamics and
58 energy production (Bertolin et al., 2018). Thereby, in collaboration with Inscoper
59 (www.inscoper.com), we have developed the control solution of our time-gated FLIM
60 microscope (fastFLIM) (Leray et al. 2013). Finally, we present the optimization of this system
61 to screen a 96-well plate with cells expressing the AURKA biosensor, together with the
62 automated process to analyse data.

63 **Material and Methods**

64 *Cell culture*

65 U2OS cells free from mycoplasma were purchased from American Type Culture Collection
66 (ATCC, HTB-96) and were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma-
67 Aldrich) supplemented with 10% fetal bovine serum (Life Technologies, Thermo Fisher
68 Scientific), 1% L-glutamine (Life Technologies, Thermo Fisher Scientific) and 1% penicillin-
69 streptomycin (Life Technologies, Thermo Fisher Scientific). Cells were cultivated at 37°C and
70 5% CO₂. The generation of GFP-AURKA and GFP-AURKA-mCherry stable cell lines were
71 generated by transfecting U2OS cells with X-tremeGENE HP transfection reagent (Roche),
72 following the manufacturer's indications. Stable clones were selected in DMEM supplemented
73 with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin and 500 µg/ml
74 Geneticin (Invivogen). Stable and transient transfections were performed with X-tremeGENE
75 HP transfection reagent (Roche) according to the manufacturer's instructions.

76 *Cell preparation for microscopy*

77 Mitotic cells were obtained after synchronisation at the G2/M transition with 100 ng/ml
78 nocodazole (Sigma-Aldrich) for 16h at 37°C. Cells were washed twice with 1X PBS and
79 incubated with prewarmed medium for 30 min to reach metaphase. For inhibitor screening, 47
80 molecules provided by KISSf (Roscoff) were used. Each molecule was used as duplicate in a
81 96-well plate and cells were blindly incubated with molecules at 1 µM for 30 minutes at 37°C.
82 Cells were washed with PBS 1 X and were fixed with paraformaldehyde 4% (from
83 paraformaldehyde 16% (Electron Microscopy Sciences), diluted in PBS) for 15min at room
84 temperature. Cells were washed three times with 1X PBS and were kept in PBS at 4°C or
85 directly used for microscopy. All microscopy experiments were performed in Nunc Lab-Tek II
86 Chamber slides (Thermo Fisher Scientific) or in 96-well plate glass cover (Zell-kontak).

87 *FLIM microscopy*

88 The fastFLIM microscope is presented in details in Results section. Briefly, the time-gated
89 FLIM system is constituted of a White light laser (Fianium sc400) and an home-made
90 wavelength selector for excitation source, a spinning disk microscope with Yokogawa confocal
91 head (CSU X1) and Leica microscope (DMI8) with 20x Leica water immersion (NA = 0.7),
92 and a fast time-gated intensifier (Picostar Lavisision using Kentech instrument HRI) optically
93 coupled to a CCD camera (Coolsnap HQ Photometrics) for wide-field detection. The system is

94 completely controlled by Inscoper hardware and software solution in order to provide HCS-
95 FLIM protocols for screen acquisitions.

96 *Data and statistical analysis*

97 Home-made automated protocol was used for lifetime calculation and cell segmentation using
98 ImageJ macro (see details in Results section). The code is available through github at
99 <https://github.com/FloIGDR/Macro-ImageJ/blob/master/Screening%20analysis>.

100 The Mann-Whitney test was used to compare the fluorescence lifetime between GFP-AURKA
101 and GFP-AURKA-mCherry (Fig 1.C, Fig 5.A).

102 **Results and discussion**

103 *Time-gated FLIM microscopy for fast cellular measurements of the AURKA FRET* 104 *biosensor in a large field of view*

105 We previously described our microscope setup for rapid FLIM acquisition (the fastFLIM
106 microscope) (Leray et al., 2013). This system can image and measure the fluorescence lifetime
107 of any fluorescent protein with a high frame rate in comparison to conventional techniques such
108 as TCSPC. Besides, based on a new method for controlling a plurality of functional modules
109 (Roul et al., 2015), the Inscoper company has recently developed a control solution for
110 microscopy systems used in life science. The solution includes an electronic device embedding
111 a microcontroller to autonomously execute sequences of microscope driving commands and an
112 application software offering the human-machine interface. The advantages of this solution are
113 the user-friendly interface and significantly increased acquisition speeds. To further increase
114 the speed capabilities of the fastFLIM microscope, we have developed an implementation of
115 the Inscoper control system to be conjugated with the microscopy setup. The system is
116 represented in Fig. 1A. Before the acquisition, the Inscoper device receives the commands sent
117 by the user through the computer and will then drive autonomously and directly all the devices
118 during acquisition. The microscopy setup works using the visible spectrum of a supercontinuum
119 pulsed laser source with a frequency of 40 MHz (Fianium sc400) and filtered by a home-made
120 wavelength selector to choose any band between 420 nm and 700 nm as light excitation. This
121 latter goes through a spinning disk microscope (Yokogawa CSU X1 and Leica DMI8
122 microscope) that allows us to perform confocal microscopy in wide-field detection mode and
123 then reaches the sample. The fluorescence emission light, after passing through the dichroic
124 and the emission filter, goes through a fast time-gated intensifier (Picostar Lavision integrating
125 an HRI Kentech Instrument). The intensifier temporal gates of 2.2 ns is triggered by the

126 excitation pulse signal coming from the laser. A delay generator on the trigger signal is used to
127 shift the gate. The time-gated wide-field fluorescence image is then captured by a CCD camera
128 (Coolsnap HQ Photometrics) and further sent to the computer. During the acquisition process,
129 on top of the spinning disk, the microscope status, and the CCD camera, the Inscoper box drives
130 the delay generator to produce different time-gated images. When using GFP fluorescence,
131 whose lifetime is around 2.5 ns, we performed five time-gated images of 2.2 ns with a
132 consecutive shift of 2.2 ns to produce adjacent temporal gates (Fig. 1B). The stack of five time-
133 gated images covers the whole fluorescence decay of the GFP. From the intensity of these
134 images, using a discrete equation to compute the temporal mean, the computer gets the mean
135 fluorescence lifetime pixel by pixel, and ultimately the FLIM image. The strength of this
136 approach lies in getting rid of fitting methods, allowing reduced photon budget (Leray et al.,
137 2013). The application software then displays the FLIM image in real time for easy-to-use
138 experiments.

139 We have recently designed a FRET-based biosensor for AURKA that allows us to track
140 AURKA activation in living cells (Bertolin et al., 2016). This biosensor is composed of the
141 whole kinase flanked by a GFP and a mCherry (hereby GFP-AurKA-mCherry) and is stably
142 expressed at physiological levels in U2OS cells. In these cells, GFP-AURKA-mCherry shows a
143 decrease in GFP lifetime of nearly 100 ps compared to the donor alone (GFP-AURKA). This
144 decrease in lifetime is indicative of FRET, and we have shown that this 100 ps downshift
145 corresponds to the difference between activated or inactivated kinase. The use of this biosensor
146 has been validated in living cells using a 63x objective with oil immersion and high numerical
147 aperture to image one single cell per image. However, this approach is not suitable for the
148 screening of AURKA activation in 96-well plates, as mitosis in these cells occurs every 24h
149 and it lasts for 1h30 only. Thus, we have decided to switch from live cells to fixed cells to
150 overcome this issue. We synchronized these cells at mitosis, and we fixed them using
151 paraformaldehyde. We then changed the objective from a 63x oil immersion to a 20x water
152 immersion (NA = 0.7) in order to image several mitotic cells in every field of view (Fig. 1C).
153 Concerning the biological sample, we verified that the fixation does not abolish FRET
154 detection. On fixed cells, we measured a difference of 100 ps between GFP-AURKA and GFP-
155 AURKA-mCherry, which corresponds to the FRET previously observed in living cells either
156 with the fast-FLIM or by Time-Correlated Single Photon Counting (TCSPC). Together, we are
157 able to use fast acquisition of FRET-FLIM images in a large field of view. This allows to

158 increase the number of the mitotic cells detected per acquisition and without losing FRET
159 efficiency although we lose some spatial resolution.

160 ***Inscoper: an embedded control software for fast acquisition in fluorescence microscopy***

161 The Inscoper imaging suite was grounded on separating the microscope and associated devices
162 control from the user interface (Fig.2). The Inscoper electronic unit performs the control of all
163 the devices around the microscope during the image acquisition. Thus, for the fast FLIM
164 system, the Inscoper box ensures bidirectional communication with all motorised devices. In
165 contrast, the Inscoper interface, run on a PC, is only used to set up the acquisition, to retrieve
166 the images from the camera and later to visualize them. The Inscoper box offers the advantage
167 of running the driving sequence, customised to the very experiment, without an operating
168 system and this avoids in particular reliability issue related, e.g. to OS update as often found
169 when using conventional acquisition software. Furthermore, since the driving code is embedded
170 on a microcontroller, it performs in real time. Overall, it allows rapid and stable communication
171 with the devices keeping full synchronisation of all the signals and ensures highly reproducible
172 experiments. The Inscoper interface gives the user access to many settings for multidimensional
173 experiments, such as timelapse, multi-channel, Z-series and multi-positions ones. Regarding
174 the fastFLIM, when the user starts a FLIM acquisition, the Inscoper box controls the
175 illumination system, the focus of the microscope, the delay generator to select the shift of the
176 delay gate at 2.2 ns, and the stage for each new XY position. These parameters can be manually
177 adjusted when the user starts a new FLIM acquisition. Among the parameters controlled by the
178 Inscoper box, only the image data goes directly to the computer from the camera. Furthermore,
179 the Inscoper box uses a bidirectional communication with the camera based on multiple trigger
180 signals. Doing so, we tightly synchronize the camera frame grabbing and the configuration
181 changes of the microscope and associated devices. In particular, before sending the trigger to
182 the camera to grab a new image, the Inscoper box waits for a trigger ready signals from the
183 camera and « new configuration reached » from the devices. A third signal called *exposure out*
184 notify the other devices that the camera is in readout step.

185 ***Automatised screening coupled with autofocus to acquire mitotic cells expressing the***
186 ***AURKA FRET biosensor within a 96-well plate***

187 We aim to perform an automated screening on a 96-well plate using the fastFLIM microscope.
188 We have developed the necessary plugins within the Inscoper microscope-control application
189 to overcome issues cause by screening methodology (Fig. 3A). They include a randomised

190 multi-position mode for each independent well, and autofocus. The design of the software
191 interface is presented in Fig. 3B. Given that the fastFLIM microscope stage is motorized and
192 controlled by the Inscoper box, the features of a 96-well plate such as the length, the width, the
193 size of the well and the distance between the wells were uploaded to allow the stage to
194 automatically browse the entire plate for the multi-position mode. The user can then choose to
195 screen the entire plate or definite number of wells. In addition, the user can also choose to image
196 multiple positions in each well. The positions within the different wells are randomly
197 determined to up to hundreds of positions for each well. In order to avoid overlap of the different
198 fields of view, a minimal distance between each position can be defined. For example, using
199 the 20x objective, we determined a minimal distance of 250 μm between each position which
200 corresponds to the size of the field of view, and we set this parameter directly in the interface.
201 After defining the above-mentioned parameters, all the positions for every selected well of the
202 96-well plate are calculated to use the Inscoper solution as for conventional multi-position
203 imaging. Concerning autofocus, it addresses a major challenge when screening long distance
204 multi-position in cellular microscopy. Although manufacturers develop the flattest plates
205 possible, a slight variation of a few μm between wells exists and leads to a focus loss.
206 Furthermore, depending on the application, the focus of interest (z position of the cell) can be
207 different from well to well. To overcome this issue, several strategies exist (Qiu et al., 2013).
208 We adapted an autofocus method based on the analysis of sharpness on a z-stack of images. In
209 further details, the user chooses the number and the size of the z-steps being analysed. For each
210 step, an image of fluorescence intensity is acquired and a 3x3 median filter is applied. Then, a
211 sharpness score is calculated for each pixel, and an additive sharpness score is determined for
212 the image. This score is used to determine the z-position of the best focus. Different channels
213 can be used for acquisition and autofocus so that any excitation lights and cell labelling can be
214 used. This also enables the user to avoid photobleaching and phototoxic effect related to the
215 labelling used for the FLIM. In the experiment presented here, we observed that with a
216 minimum illumination of GFP, we were still able to find the best focal plane of cells expressing
217 GFP-AURKA and GFP-AURKA-mCherry and this without perturbing the GFP fluorescence
218 lifetime. This is why we decided to use the GFP illumination for the autofocus procedure as
219 well.

220 ***An automatized data analysis pipeline for screening: cell-by-cell fluorescence lifetime***
221 ***determination***

222 Depending on the number of positions for each well, the number of images can vary from
223 hundreds to thousands, calling for automatically analysing the data. Since a single frame
224 contains multiple cells, we segmented the image to compute cell-wise measurements later. Our
225 data analysis pipeline (Fig. 4) stores the time-gated stack of fluorescence images, the
226 corresponding fluorescence lifetime images, and the cropped images of segmented cells
227 together with a table summarizing the available measurements for this cell. At first, we compute
228 the FLIM images in a post-acquisition step. The advantage is to adjust the parameters along the
229 process and have the ability to recompute the mean lifetime posteriorly, an important feature to
230 tweak, e.g. the pixel threshold value of the first time-gated image. Indeed, this latter value limits
231 the extent of the fluorescence lifetime computing, as pixels below the threshold do not have
232 sufficient photon count to let us compute a reliable lifetime. In our case, a threshold at 3000
233 grey levels appears suitable (Leray et al., 2013). The computing is performed by an *ad hoc*
234 ImageJ macro in which the user is asked to provide the size of the intensifier gate (gatewidth),
235 the step between the different time-gated images (stepdelay) and the threshold value (grey
236 value). In a second step, we segment the cells using the first time-gated intensity image, which
237 harbours the highest intensities. To do so, we performed a Gaussian blur, followed by an
238 automatic threshold in ImageJ. The corresponding parameter and threshold-determining
239 algorithm are set by the user prior to batch processing. In our case of cells expressing the
240 AURKA biosensor and synchronized at mitosis, a Gaussian blur provided by ImageJ using a 1-
241 pixel radius circular neighbourhood and the default threshold-determining algorithm offer the
242 highest accuracy possible. Finally, the watershed algorithm separates adjacent cells. Different
243 regions of interest are then determined. The lifetime image is then broken into cropped sub-
244 frames containing each a single cell and saved in separated files together with a file indicating
245 the mean fluorescence lifetime over the cell, the standard deviation and cell-area in pixels. It
246 allows controlling the quality of the segmentation and further statistical analyses.

247 ***Validation of a 96-well plate screening using AURKA FRET biosensor***

248 To validate our screening process, we investigated a 96-well plate containing U2OS stable cell
249 lines expressing GFP-AURKA or GFP-AURKA-mcherry (Fig. 5). We seeded the same amount
250 of cells in each well and synchronised them in mitosis using nocodazole. This is known to
251 induce a peak of AURKA expression (Nikonova et al., 2013) and correspondingly a peak of
252 signal from the biosensor. Then, cells were released in normal medium for 30 min and reach

253 metaphase. The use of a multi-channel pipette allowed a homogenous synchronization
254 throughout the 96 wells of the plate, and it ensured a rapid washout of the culture media in all
255 the wells in less than one minute. We then used 4% paraformaldehyde to fix cells in metaphase
256 as shown in Fig. 1. We screened the plate with a 20x water immersion objective, as oil
257 objectives have front oil problems during stage motions preventing proper imaging.
258 Furthermore, we also used a water dispenser (Leica) to overcome water evaporation and to
259 ensure a perfect immersion throughout the screening. All acquired images were analysed as
260 described previously (Fig. 4). The resulting fluorescence lifetimes are plotted as a heat map for
261 each well (Fig. 5A, left panel). All cells within a well do not display the same number of pixels
262 with photon count above the threshold (see analysis section). To avoid an imbalance, we
263 weighted the lifetime from each cell by its area in pixels and the standard deviation for each
264 cell before to calculate the mean lifetime for each well. Such a heat map showed a correct
265 accuracy allowing to clearly distinguish between wells with GFP-AURKA and GFP-AURKA-
266 mCherry. When pooling the fluorescence lifetimes from both conditions (Fig. 5A, right panel),
267 we found a significant difference of about 90 ps, similar to FRET measurement in single-cell
268 experiments (Fig. 5B).

269 To go further, we implemented a blind screening test on 47 molecules provided by KISSf
270 (Roscoff). These molecules were chosen for their ability to inhibit Aurora B kinase activity by
271 classic in vitro kinase assay. Each molecule's name referred to the well coordinates within the
272 96-well plate. We incubated cells expressing GFP-AURKA-mCherry with these molecules at
273 1 μ M for 30 minutes at 37°C and fixed the cells before screening following our methodology
274 (Fig 5.C). The results showed that several molecules were potential inhibitors of AURKA, as
275 the fluorescence lifetime significantly increased for molecules A6, A7, D5-7, E6, G7, H5 and
276 H6 when compared to DMSO. Interestingly, these results were strengthened by the revelation
277 that compound H5 was MLN8237, a common inhibitor of AURKA. These results demonstrate
278 the potentiality of our biosensor system as an automated tool for molecular screening purposes.

279 **Conclusion**

280 We described a method to perform automated screening with FLIM using our time-gated
281 fastFLIM microscope. This microscope uses temporal gates to selectively acquire sections of
282 the fluorescence decay after each pulsed laser. Our system is controlled by the Inscoper imaging
283 suite, which allows getting rid of the latency caused by the operating system. Taking advantage
284 of its speed, we improved it to automatically screen 96-well plates. Furthermore, we

285 implemented multi-well randomized acquisitions modules with an autofocus module. We also
286 developed a pipeline to automatically analyse thousands of images and obtain fluorescence
287 lifetime data for each cell. Importantly, the software can rapidly generate an image of
288 fluorescence lifetime without any fitting. We validated this methodology by screening the
289 FRET efficiency using our AURKA biosensor. We now aim to screen for novel compounds
290 and find new and potentially more potent drugs. Furthermore, this methodology can also be
291 used to characterize these drugs and the cellular phenotype they induce, through the
292 fluorescence images obtained. To date, few High Content Analysis (HCA)-FLIM systems have
293 arisen. These generate time-gated windows with a pulsed laser, but fitting to determine
294 fluorescence lifetime is necessary (Görlitz et al., 2017) which does not permit to automate the
295 analysis. These tools were recently used to investigate protein-protein interactions in an HCA
296 mode (Guo et al., 2019; Margineanu et al., 2016). We here demonstrated that using biosensors
297 and specially kinase activity biosensor increases the information that can be obtained from
298 screening procedures. Combined to our recent multiplex systems (Demeautis et al., 2017) it
299 will allow to track the activity of several biosensors at the same time and broaden the impact of
300 HCA-FLIM to provide more insight into kinase activity.

301

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319 **Competing interest**

320 O.B. is the CTO of Inscoper Company, a spin-off of the IGDR. J.P. and M.T. are scientific
321 advisors of Inscoper. All other authors have no competing interest.

322 **Author contributions**

323 F.S. designed, performed and analysed the experiments; G.L., J.P. and O.B developed tools and
324 methods and; M.T. conceptualised the study, coordinated the work and provided funding. F.S.
325 and M.T. wrote the paper with the input of all authors.

326

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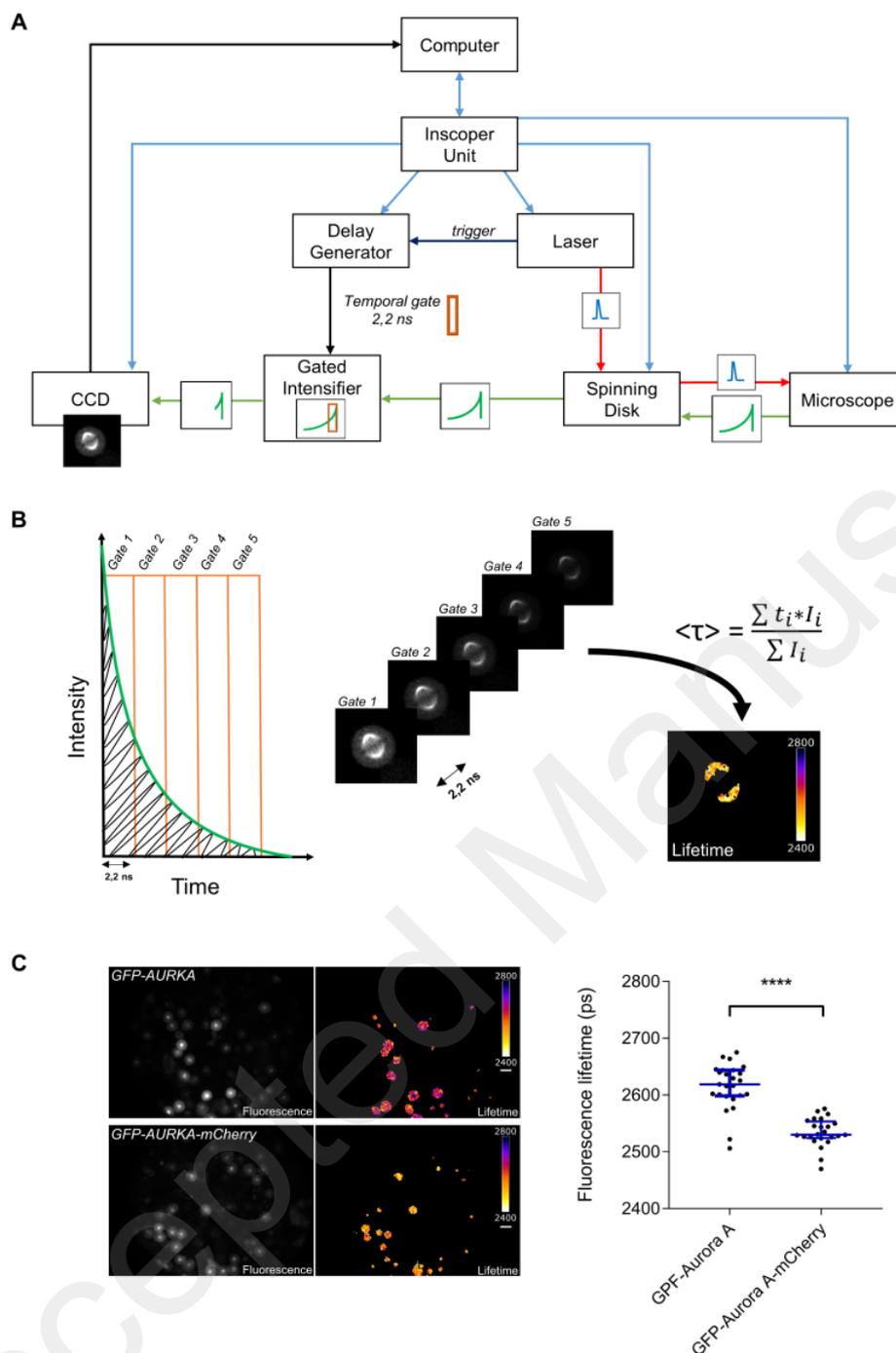
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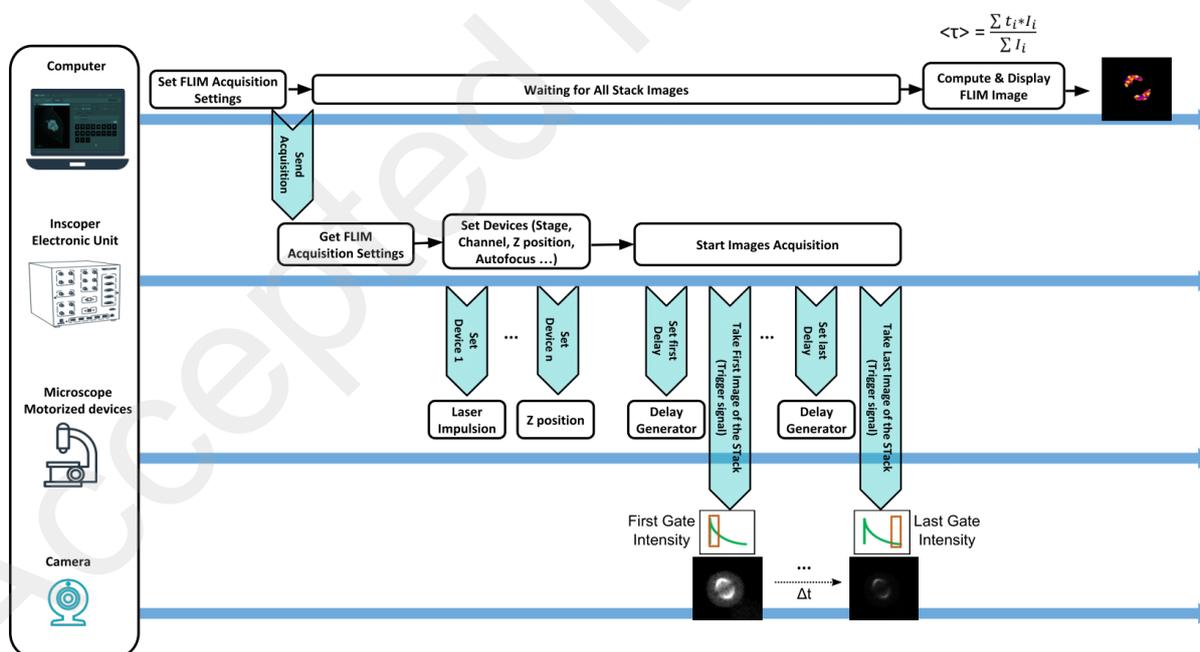


400

401 **Fig. 1. The fast-FLIM microscope can measure FRET efficiency of the AURKA biosensor**
 402 **in fixed cell lines. A.** Model illustrating the mode of action of the fast-FLIM. The computer
 403 sends commands to the Inscoper electronic unit, which in turn communicates with the other
 404 devices. For each laser pulse, a trigger from the laser is sent to the, intensifier generating a gate
 405 of 2.2 ns during which the fluorescence emission is captured. The delay generator is used to
 406 position the gate at the desired timing during the fluorescence decay. After intensifying the
 407 fluorescence signal of the selected temporal gate, a CCD camera is used to acquire the time-

408 gated image and is sent to the computer. **B.** Model illustrating the generation of one
 409 fluorescence lifetime image using the AURKA biosensor in U2OS cells. A stack of five time-
 410 gated images are acquired sequentially after controlling the delay generator to position the gate.
 411 Each image corresponds to a juxtaposed gate of 2.2 ns to cover the whole fluorescence decay.
 412 From the intensity of the five images, the pixel by pixel mean fluorescence lifetime is calculated
 413 using a discrete temporal mean of the decay to form the fluorescence lifetime image. **C.** The
 414 fast-FLIM is suitable to measure FRET efficiency of the AURKA biosensor in fixed cells. (Left
 415 panel) On the left, representative fluorescence (GFP channel) of U2OS cells expressing GFP-
 416 AURKA or GFP-AURKA-mCherry, synchronized at mitosis, fixed with 4% paraformaldehyde
 417 and imaged with a 20x objective (NA = 0.7). On the right, the corresponding fluorescence
 418 lifetime images. Pseudocolour scale correspond to the mean lifetime. Scale bar, 30 μ m. (Right
 419 panel) Corresponding scatter plot of the fluorescence lifetime; Each dot corresponds to the
 420 fluorescence lifetime of one cell. n=20-30 cells per condition from three independent
 421 experiments. Blue bars represent median and interquartile. ***P<0.0001 against the 'GFP-
 422 AURKA' condition. Statistical test: Wilcoxon-Mann-Whitney test.

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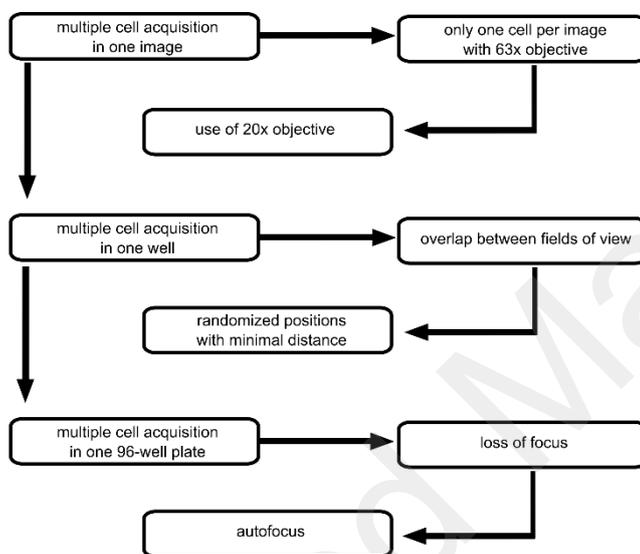
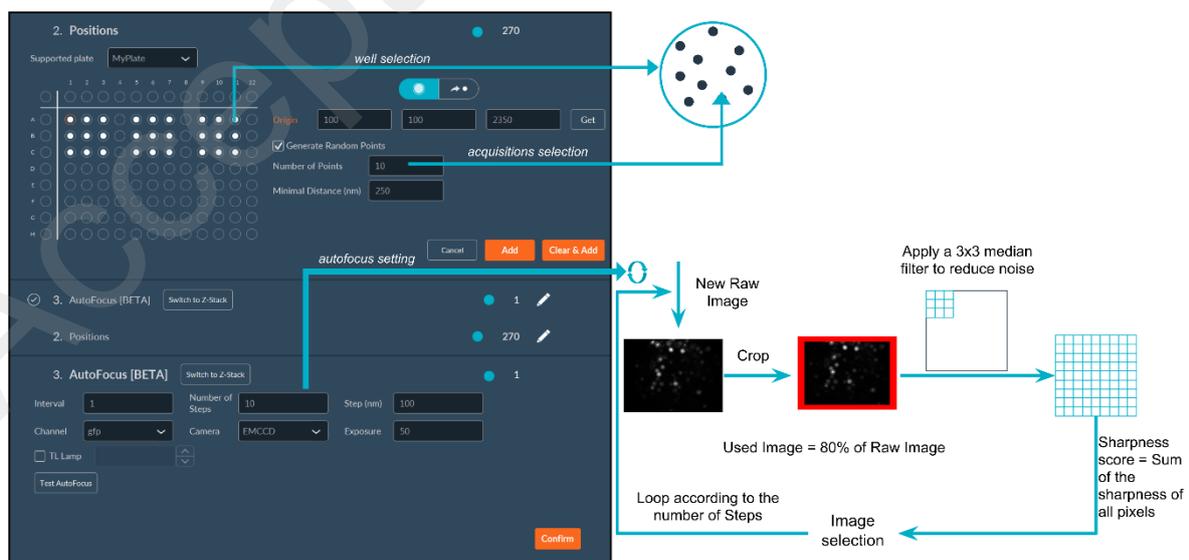


424

425 **Fig. 2. Hardware and Software features for the acquisition of a FLIM image.** From right
 426 to left and from top to bottom: from the Inscoper graphical user interface on the computer, the
 427 user set FLIM acquisition sequence which is sent to the Inscoper electronic unit. While
 428 computer wait for the acquired images, the Inscoper unit analyses the sequence parameters and

429 set the different devices by communicating to the different microscope devices. Then the
 430 electronic unit starts the acquisition of the stack of time-gated images by alternatively setting
 431 the delay generator and triggering the camera. All the camera images were then transferred
 432 to the computer and when the stack is completed, the computer calculate and display the FLIM
 433 image. In the same time, the Inscoper electronic unit re-start a new FLIM acquisition after
 434 setting the different devices if necessary as it was previously planned by the user at the
 435 beginning of the acquisition sequence. The process is reproduced as many times as necessary
 436 to complete the full sequence.

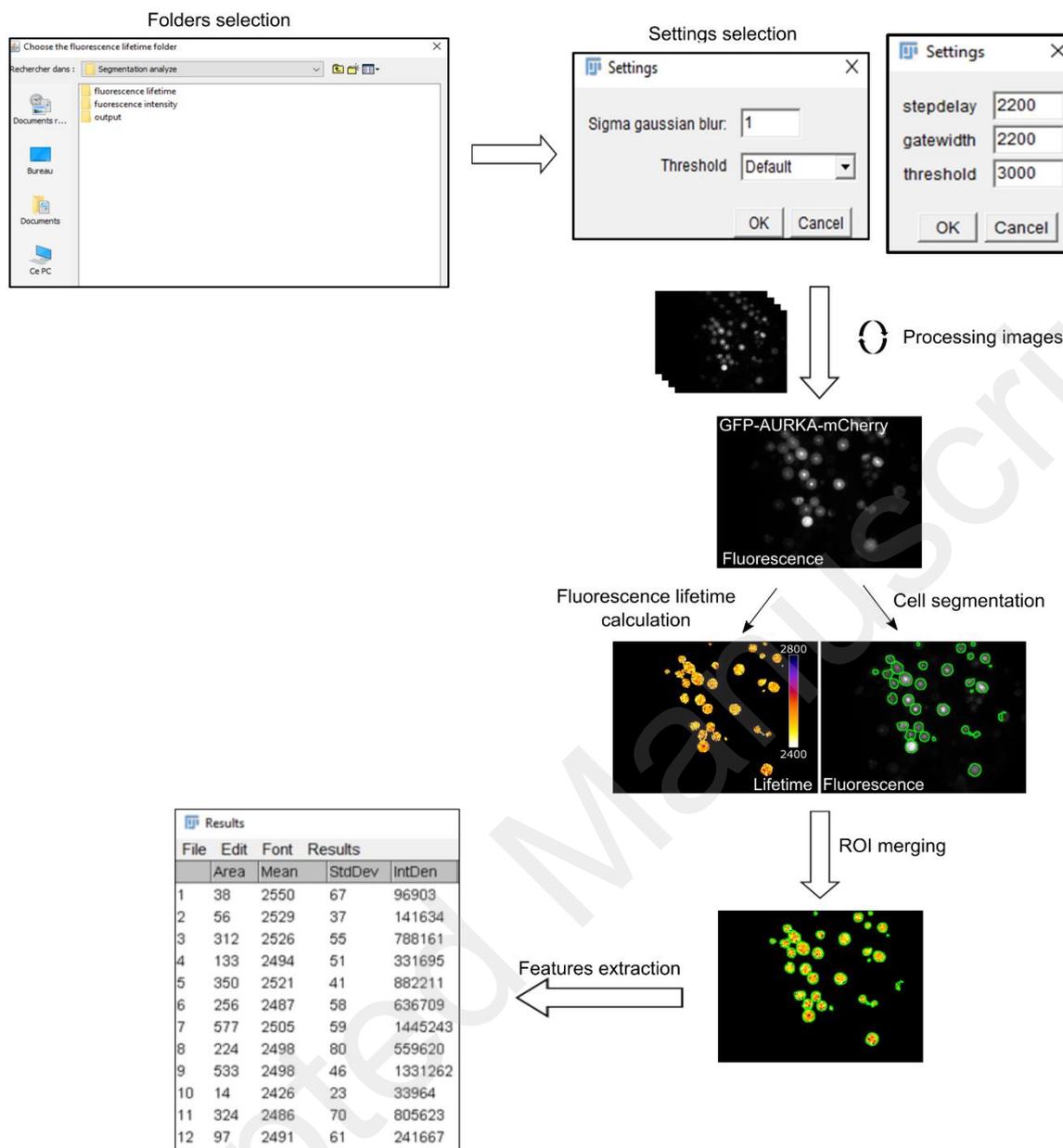
437

A**B**

438

439

440 **Fig3. Multi-well and autofocus modules of the Inscoper user interface for screening**
441 **applications. A.** Workflow diagram of the screening strategy. **B.** Inscoper User Interface. At
442 the top left of the windows is presented the scheme of the 96 multi-well plates. The user
443 determines the wells of interest (where the acquisition will be carried out) by individual well,
444 by line or by column. Then the user determines the number of random points for each well and
445 the minimum distance between each position (as presented in the scheme at the top right). With
446 these information, the interface calculates the coordinates of each point based on the initial
447 calibration of the stage. At the bottom of the windows is presented the autofocus panel which
448 permits to determine the optimal z plane for each xy position. The user determines the interval,
449 the number of steps, the step size and the color channel used for autofocus. The algorithm then
450 calculates the best focus by calculating a sharpness score for each acquired image (see Results
451 section for details). The plan with the highest score is selected. To speed up the execution of
452 the autofocus, it is possible to define a dedicated exposure time different to the FLIM images.



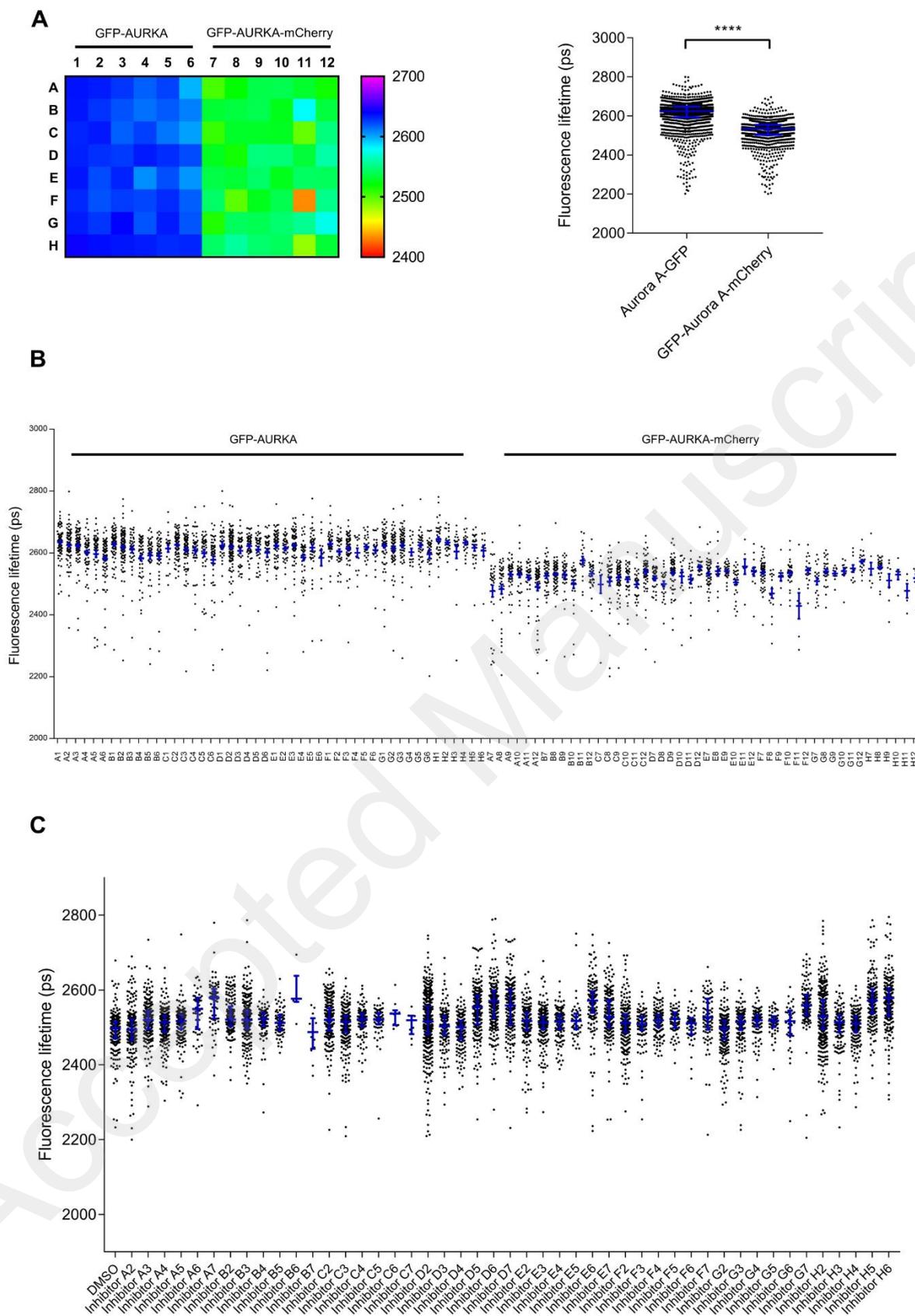
453

454 **Fig. 4. Batch mode analysis by cell segmentation.** Analysis of fluorescence images of U2OS
 455 expressing GFP-AURKA-mCherry using an ImageJ home-made macro. From top to bottom:
 456 the user first chooses the folder containing fluorescence images, and the two destination folders
 457 for the calculated lifetime image and for the segmented intensity images with a file containing
 458 all the values of the features for each ROI. From the stack of time-gated images, the
 459 fluorescence lifetime image is generated using the threshold, size of the gate and step values
 460 settled by the user. From the first time-gated fluorescence image, the cells are segmented
 461 according to the settings defined by the user. This segmentation is applied on the fluorescence
 462 lifetime image and for each ROI (cell) is calculated the mean fluorescence lifetime, the number

463 of pixel (area of the ROI) and the standard deviation between the pixel values. This analysis is
464 automatically applied for all the images coming from the chosen folder.

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467 **Fig 5. Multiwell plate screening of AURKA biosensor. A.** (Left panel) Heatmap of the mean
 468 fluorescence lifetime values weighted by the area and the standard deviation in each well of a

469 96-well plate seeded with U2OS cells expressing GFP-AURKA and GFP-AURKA-mCherry.
470 Cells have been synchronized at mitosis and fixed with PFA before being screened with the
471 HCS-FLIM. Pseudocolour scale: lifetime values in ps. (Right panel) Corresponding scatter plot
472 of the fluorescence lifetime values coming from all the wells expressing GFP-AURKA or GFP-
473 AURKA-mCherry. Each dot represents lifetime of one cell and blue bars represent median and
474 interquartile. ****P<0.0001 against the GFP-AURKA condition. Statistical test: Wilcoxon-
475 Mann-Whitney test. **B.** Scatter plot of fluorescence lifetime values of each cell sorted by well
476 (from A1 to H12). Each individual point represents a single cell. Bars represent median and
477 interquartile. **C.** Scatter plot of fluorescence lifetime values of each cell sorted by inhibitor
478 treatment. Each individual point represents a single cell. Bar represent median and interquartile.