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1 **Live-cell imaging and mathematical analysis of the** 2 **“community effect” in apoptosis**

3

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6

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14 # Equal contribution.

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31 **Abstract**

32 As a cellular intrinsic mechanism leading to cellular demise, apoptosis
33 was thoroughly characterized from a mechanistic perspective. Nowadays
34 there is an increasing interest in describing the non-cell autonomous or
35 community effects of apoptosis, especially in the context of resistance to
36 cancer treatments. Transitioning from cell-centered to cell population-relevant
37 mechanisms adds a layer of complexity for imaging and analyzing an
38 enormous number of apoptotic events. In addition, the community effect
39 between apoptotic and living cells is difficult to be taken into account for
40 complex analysis. We describe here a robust and easy to implement method
41 to analyze the interactions between cancer cells, while under apoptotic
42 pressure. Using this approach we showed as proof-of-concept that apoptosis
43 is insensitive to cellular density, while the proximity to apoptotic cells
44 increases the probability of a given cell to undergo apoptosis.

45 **Introduction**

46 Inconspicuous at the single cell level, apoptosis is a powerful
47 mechanism when triggered at the organismal level. It is the sculpting force
48 behind all multicellular organism morphogenesis, ensuring all organs have the
49 right size, supernumerary cells are removed and neurons innervate the
50 correct targets.

51 In cancer, apoptosis is an efficient guardian against oncogenic
52 transformation and it is the preferred mechanism employed by tumor
53 suppressor proteins to eliminate dangerous rogue cells (1). For instance, p53
54 can trigger apoptosis by up-regulating the expression of several pro-apoptotic
55 proteins (1). Apoptosis is undeniably the main executioner of most cancer
56 drugs and radiotherapy (2).

57 Apoptosis is triggered when one of two distinct pathways is engaged:
58 the extrinsic or the intrinsic. Extrinsic apoptosis relies on the stimulation of a
59 death receptor-family member (e.g., TRAIL-R, FAS or TNF-R) by a TNF-
60 related cytokine, and activation of caspase 8 and 10 via the death-inducing
61 signaling complex (DISC) (3). The intrinsic pathway is initiated by an
62 intracellular death stimulus such as DNA damage, chemo- and radiotherapy

63 or tumor suppressor activation. In this case, the point of no return is
64 mitochondrial outer-membrane permeabilization (or MOMP) followed by
65 cytochrome *c* release, apoptosome assembly and activation of caspase 9 and
66 then 3 and 7. Mitochondrial permeabilization is tightly regulated by the BCL-2
67 family proteins that can be anti-apoptotic (BCL-2, BCL-xL or MCL-1), pro-
68 apoptotic (BID, BIM, BAD, PUMA or NOXA) and effector pore-forming
69 proteins such as BAX or BAK (for review see (4)).

70 Once apoptosis is triggered, this does not inevitably lead to cell death,
71 as it is now accepted that cells can survive if mitochondrial permeabilization
72 does not affect all mitochondria or if the ESCRT-III complex restores plasma
73 membrane integrity (5) (6) (7). Currently, there is a lack of understanding of
74 how and why the kinetics of apoptosis varies so much between cells or
75 following different treatments.

76 The heterogeneous response to apoptosis is also described in isogenic
77 cancer cell lines. This is puzzling since these cells are genetically identical
78 and should express similar levels of both pro- and anti-apoptotic proteins.
79 Cellular heterogeneity to undergo apoptosis thus appears to originate from
80 non-genetic transcriptional variability. This translates into cell-to-cell variations
81 of pro- and anti-apoptotic protein expression and activity. For instance, the
82 Lahav group elegantly showed that there is a high variability of p53 response
83 to DNA damaging agents; apoptotic cells accumulate p53 much faster and
84 earlier, while this accumulation is delayed in resistant cells, allowing time for
85 the up-regulation of pro-survival IAP (inhibitor of apoptosis) proteins (8).

86 Recent publications shed light on the effects apoptotic cells exert on
87 neighboring cells. They can actively release EGFR ligands, FGF2 or Wnt3 to
88 boost the survival and proliferation of neighboring cells (9) (10) (11). This
89 paracrine crosstalk is also complemented by mechanotransduction
90 mechanisms triggered by tissue stretching around apoptotic cells, which
91 involves the Yes-associated protein (YAP) pathway (12).

92 There is therefore an increasing need to accurately estimate the
93 variability of apoptosis induction in a given cell population. To achieve this, we
94 developed a medium-throughput imaging pipeline using apoptotic cell
95 markers, coupled with mathematical analysis. This allowed us to unveil a
96 neighboring effect for apoptosis induction, irrespective of cell death stimuli or

97 cell density, whereby the probability of undergoing apoptosis increases in the
98 proximity of apoptotic cells. Hence, the combined application of imaging and
99 computational analysis to evaluate the response to a given apoptotic stimulus
100 may provide new insight into misunderstood phenomena such as fractional
101 killing or apoptosis-induced proliferation, which are major concerns in the
102 context of apoptosis-based cancer therapies.

103 **Results**

104 *Live-cell imaging system to trigger and measure multi-stimuli apoptosis*

105 To mathematically analyze the kinetics of apoptotic cell death while
106 varying different parameters, we first set up a cellular system allowing
107 medium-throughput quantification of apoptosis at the cell population level. For
108 this, we used melanoma WM115 cells stably expressing mCherry-tagged
109 histone H2B (H2B-mCherry) to spatially identify the exact topology of a given
110 cell (**Figure 1A**). Following treatment with an apoptotic stimulus, the induction
111 of apoptosis is easily visualized using the live cell-impermeant dye SYTOX
112 Green (SG). Live cells can thus be identified by the nuclear-localized H2B-
113 mCherry-only signal (red nuclei), whereas apoptotic cells appear yellow,
114 owing to the colocalization of H2B-mCherry and green SG (**Figure 1A**). Next,
115 we used various stimuli to trigger apoptosis. Treatment with TNF α and
116 cycloheximide (CHX) was initially used as a model for death receptor-
117 mediated apoptosis. Of note, CHX enhances TNF α -induced apoptosis by
118 blocking the translation of short-lived anti-apoptotic proteins (13). Downstream
119 of the TNF α receptor 1/2 (TNFR1/2), the DISC activates caspase 8 and
120 triggers MOMP via BID cleavage (**Figure 1B**). To induce intrinsic apoptosis,
121 we used two death stimuli: first, a combination of BH3 mimetics ABT-737
122 inactivating the anti-apoptotic proteins BCL2, BCL-xL and BCL-w, and UMI-77
123 targeting MCL1; second, a doxycycline (dox)-inducible WM115 cell line to
124 activate the mitochondrial pore-forming protein BAX (**Figure 1B**). These
125 apoptotic stimuli were then applied to SG-pre-loaded WM115 H2B-mCherry
126 cells plated at different densities and imaged periodically (1 or 2 hours
127 intervals) using the IncuCyte live-cell imager (**Figure 1A**).

128 Next, we validated the induction of apoptosis by assessing the
129 processing of effector caspase-3 into p17/p19 fragments and the cleavage of
130 PARP1, which is a hallmark of apoptosis effectiveness. As shown In **Figure**
131 **2A** and **B**, treatment with TNF α /CHX (TC), BH3 mimetics (BH3m) and
132 increasing doses of doxycycline efficiently induced apoptosis. Of note,
133 caspase-3 processing and PARP1 cleavage were blocked by treatment with
134 the pan-caspase inhibitor Q-VD-OPh. In addition, IncuCyte-based live-cell
135 imaging was used to delineate the kinetics of apoptosis triggered by the
136 different stimuli described above (**Figure 2C, D**). Apoptotic cells were marked
137 by SG and quantified over time, with image acquisitions every 1-2 hours over
138 a period of 48 hours on average. **Figure 2E** shows this imaging method can
139 efficiently detect and discriminate between living cells (mCherry-positive) and
140 apoptotic cells (SG-positive). The figure presents results of TNF α /CHX and
141 BH3 mimetics treatment, albeit the same was observed for WM115 cells
142 overexpressing BAX (data not shown).

143 Using this cell model of apoptosis induction, coupled with medium-
144 throughput live-cell imaging we therefore established an imaging technique
145 able to monitor the heterogeneous induction of apoptosis at the cell
146 population level.

147

148 ***Cell clustering has no effect on the incidence of apoptosis***

149 Following different pro-apoptotic treatments (**Figure 1B**) and IncuCyte-
150 based live cell imaging, we obtained datasets of H2B-mCherry images (red
151 images) and the corresponding SYTOX Green staining (green images) at
152 different time points, for a kinetics analysis of 48 hours. We then designed two
153 independent pipelines to segment H2B-mCherry-marked nuclei, one based on
154 the CellProfiler software and the other written in Python, using standard image
155 libraries to ensure a robust analysis (**Figure 1A**). The analysis pipeline
156 included the following steps. First, for a pair of red/green images the output is
157 a segmented “red” image with a known red object centroid position (X_i , Y_i)
158 onto which a measurement of the green signal is superposed (I_i , representing
159 the maxima green signal intensity) (**Figure 3A**). Second, for a given time-point
160 t , the analysis pipeline retrieves a set of N red centroid positions (X_i , Y_i) with
161 their associated SYTOX Green intensity signal I_i . Data analysis is then

162 performed using three parameters δ , n and I_t , where δ is the inter-cellular
163 distance at which another cell(s) is/are considered to be in contact and could
164 influence each other, n is the threshold number of neighboring cells above
165 which a cell is considered to be in a cluster and I_t is the threshold intensity of
166 SYTOX Green, derived from background noise, above which the cell is
167 considered to be apoptotic. Of note, two cells are considered neighbors if the
168 distance between their centroids is below δ (**Figure 3A**).

169 Once we determined δ , n and I_t , we determined for each cell whether it
170 was apoptotic (A) or not (\bar{A}). We then established the relationships of
171 proximity between cells (the so-called neighboring effect) by determining for
172 each cell whether it was in a cluster of cells regardless of their apoptotic
173 status (C) or with a known apoptotic status (Ca) (**Figure 3B**). This method is
174 well suited to quantify spatial relations between apoptotic cells.

175 Using this approach, we were first interested in determining whether a
176 correlation between cell clustering and apoptosis (between C and A). This is
177 driven by previous work suggesting that cell density is an important modulator
178 of both extrinsic and intrinsic apoptosis (14) (15-17).

179 By examining three scenarios, in which a given cell was considered to
180 be in a cluster if it was surrounded by 1, 2 or 3 neighboring cells (regardless
181 of their apoptotic status), we found that a cell with no neighbors (\bar{C}) had the
182 same probability of entering apoptosis as a cell in a cluster (of 1, 2, or 3
183 neighboring cells) (**Figure 3C-E** for TNF α /CHX treatment). We then computed
184 and plotted the time dependent p -value of a Fisher exact test that evaluates
185 the independence between A and C (**Figure 3C-E**, bottom panels). Of note, if
186 the p -value is high (as in **Figure 3C-E**, bottom panels), this means that the
187 hypothesis that A and C are independent cannot be rejected. In other words,
188 this means that the probability of being apoptotic whenever one cell has
189 neighboring relation(s) (probability $p(A/C)$, blue curve in **Fig. 3C-E**, top
190 panels) and the probability of being apoptotic when one cell is isolated
191 (probability $p(A/\bar{C})$, orange curve) might be equal.

192 The same holds true when triggering intrinsic apoptosis with BH3
193 mimetics (**Figure 3F**) or when overexpressing the pro-apoptotic protein BAX
194 (**Figure 3G**). Though these results were obtained at low cell density, we

195 confirmed their relevance for cells grown at a high density (**Supplementary**
196 **Figure 1**).

197 Hence, these data suggest that cell clustering has no impact on the
198 occurrence of apoptosis.

199

200 *The induction of apoptosis is enhanced by the proximity of a cell to an* 201 *apoptotic cell*

202 Next, we tested whether the proximity of a cell with one apoptotic cell
203 or an apoptotic cluster (up to three apoptotic neighboring cells) was correlated
204 with its propensity to undergo apoptosis. We obtained results in complete
205 contrast to Figure 3, as the probability to enter apoptosis when in proximity
206 with one or more apoptotic cells (probability $p(A/C_a)$, blue curve) was
207 significantly higher than the probability to enter apoptosis when isolated from
208 apoptotic cells (probability $p(A/C_a)$, orange curve) (**Figure 4A-C** for
209 TNF α /CHX treatment). Indeed, the p -value (Fisher's exact test) was
210 significantly low ($p < 0.05$) for the intermediate time points, meaning that both
211 probabilities cannot be considered as equal. We observed a higher p -value at
212 the beginning and the end of the kinetics, which does not affect our analysis,
213 since there are few apoptotic cells at beginning of the time-course and, at the
214 end, most cells are apoptotic and the neighboring effect saturates. This
215 observation also holds true for treatment with BH3 mimetics and BAX
216 overexpression (**Figure 4 D, E**). Finally, we validated these data at a higher
217 cellular density (**Supplementary Figure 2**).

218 Taken together, our analysis pipeline reveals that apoptosis induction
219 is influenced by a neighboring effect: the likelihood of a cell to undergo
220 apoptosis is significantly higher when it is in proximity with apoptotic cells.

221 **Discussion**

222 Defective induction of cell death is the cause on many diseases,
223 ranging from cancer to neurodegenerative diseases, and it is therefore
224 important to study its propagative nature using appropriate tools.

225 Here, we report the development of an analysis pipeline for medium-
226 throughput imaging of apoptotic cancer cells that integrates several key

227 parameters: temporal dimension, topographic localization of both living (cells
228 stably expressing the mCherry-tagged histone 2B) and apoptotic (SYTOX
229 Green-positive) cells, while applying several apoptotic stimuli.

230 Using this approach, we found that cancer cells have a similar
231 probability of undergoing apoptosis if they are in proximity or not with other
232 cells of unknown status. In other words, cell crowding does not have a
233 protective effect against apoptosis. However, a cell is more likely to become
234 apoptotic if it is in close contact with apoptotic neighboring cells. This holds
235 true for all of the apoptotic stimuli we tested, either extrinsic or intrinsic
236 inducers of apoptosis. These results are in line with Bhola and Simon's study
237 demonstrating that daughter cells, with a high likelihood of being in proximity,
238 synchronously undergo apoptosis. This synchrony is lost as cells become less
239 related over time (18). Apoptotic synchrony is therefore transiently heritable
240 and is lost due to noise in protein translation (19). To take into account
241 progeny and monitor cell proliferation history, we would need to introduce into
242 our imaging pipeline a lineage tracer such as the one developed by Oren and
243 colleagues (20).

244 Our experimental settings are in 2D cellular cultures, yet one might
245 apply it to 3D cultures such as cancer organoids. For this, however, the
246 sample will require transparization and 3D imaging microscopy. Here, the
247 proof-of-concept is provided for apoptosis, though the protocol could easily be
248 adapted to other types of programmed cell death, such as caspase-
249 independent cell death, necroptosis or pyroptosis. Importantly, this method
250 could also be helpful in identifying factors facilitating or inhibiting cell death
251 propagation. Similar methods were developed to quantify the occurrence of
252 fractional killing, a barrier for effective cancer treatments, and helped establish
253 mediators such as the anti-apoptotic protein MCL1 (21) (22).

254 Recent research clearly demonstrates that while apoptosis is
255 immunologically silent, it still involves releasing a plethora of signaling
256 molecules that impinge on the fate of surrounding cells. The term of
257 apoptosis-induced apoptosis was coined in *Drosophila*, where apoptotic cells
258 in the wing disk triggered apoptosis in trans by secreting Eiger (the TNF
259 homologue in flies), and stimulating pro-apoptotic JNK signaling (23). The
260 same holds true in mammals during the regressive phase of the hair follicle

261 (23). In cancer, the radiation-induced bystander effect, through which
262 irradiated cells lead to the death of non-irradiated neighboring cells, is also a
263 good example of this concept (24). Interestingly, recent studies revealed the
264 rapid propagation of ferroptotic cell death, and the analysis pipeline described
265 herein might provide further mechanistic insights (25) (26). Inversely,
266 apoptosis-induced proliferation has been unveiled and describes a state in
267 which dying cells release prostaglandins and Wnt3, instructing the
268 proliferation of nearby cells (27) (11). Recently, the group of Tait showed that
269 apoptotic cells also release FGF2 to enhance the anti-apoptotic resilience in
270 trans (10).

271 The analysis method developed here could increase and enrich our
272 knowledge on “community effects” of cell death so that they can be blocked or
273 enhanced, according to the physio-pathological context.

274 **Material and methods**

275 *Cell lines*

276 Human melanoma cells WM115 (a gift from R. Insall – The Beatson Institute,
277 Glasgow, UK) were maintained in DMEM supplemented with 2 mM L-
278 glutamine (ThermoFisher Scientific, 25030-24), non-essential amino acids
279 (ThermoFisher Scientific, 11140-035), 1 mM sodium pyruvate (ThermoFisher
280 Scientific, 11360-039), 10% FBS (Eurobio, CVFSVF00-01) and 1%
281 penicillin/streptomycin (ThermoFisher Scientific, 15140-122).

282

283 *Stable cell line generation*

284 For retroviral transduction, Phoenix Ampho 293T cells (2×10^6 in 10 cm in
285 diameter Petri dishes) were transfected with pQXIN H2B-mCherry (gift from
286 Dr. Luca Fava) using Lipofectamine 2000 (ThermoFisher Scientific,
287 11668019). Twenty-four hours later, the virus-containing supernatant was
288 filtered and used to infect WM115 cells in the presence of 1 $\mu\text{g}/\text{mL}$ polybrene
289 (Sigma-Aldrich, H9268). This was repeated with fresh viral supernatant 48
290 hours later. Melanoma cells stably expressing H2B-mCherry were further
291 purified by cell sorting. WM115 H2B mCherry cells with doxycycline-inducible
292 expression of the BAX protein were created using the Sleeping Beauty

293 transposon system (28). The plasmids used were piTR1 BAX (gift from David
294 Goldschneider) and pCMV(CAT)T7-SB100 (Addgene, 34879) for the
295 expression of SB100X transposase. WM115 H2B mCherry cells were then co-
296 transfect with piTR1 BAX and pCMV(CAT)T7-SB100 plasmids using
297 Lipofectamine 2000 and selected with puromycin (1 µg/mL).

298

299 *Western blotting*

300 Cell lysates were prepared using NP-40 lysis buffer (1% NP-40, 1 mM EDTA,
301 150 mM NaCl, 50 mM Tris pH 7.4, 1 mM PMSF, Complete Protease Inhibitors
302 (Sigma-Aldrich, 4693116001)). Protein content was determined by Bio-Rad
303 assay, then 25-50 µg of proteins were separated on SDS-polyacrylamide gels
304 (Biorad) under denaturing conditions (SDS PAGE sample loading buffer
305 (VWR, GENO786-701) supplemented with 1 mM DTT) and finally transferred
306 onto nitrocellulose. Membranes were probed with the following antibodies at a
307 1/1,000 dilution unless otherwise stated: BAX (2772, Cell Signaling),
308 Caspase-3 (9662, Cell Signaling), PARP1 (9542, Cell Signaling), β-tubulin
309 (2146, Cell Signaling).

310 The nitrocellulose membranes were rinsed 3 times for 10 min in TBS-Tween
311 0.1% and then incubated with appropriate secondary antibody coupled to
312 HRP for 1 hour at room temperature. Finally, the proteins of interest were
313 detected using Clarity Western ECL (Biorad, 1705060) and chemiDoc imager
314 (Biorad, 17001401).

315

316 *IncuCyte imager-based cell viability assay*

317 Cell viability was determined using an IncuCyte Zoom imaging system
318 (Sartorius). Cells were seeded onto Imagelock 96-well plates in media
319 containing 30 nM SYTOX Green (Life Technologies, S7020). Following
320 different apoptotic treatments, the cells were imaged every 60 or 120 minutes
321 and the analysis of the SYTOX Green-positive cells was performed using the
322 available IncuCyte image analysis software (Essen Bioscience).

323

324 *Modeling the progression of apoptosis*

325 ***Data description***

326 Nuclei, set as objects, are delineated at each time step using automatic
327 segmentation of mCherry fluorescence ("Red") images via the CellProfiler
328 pipeline for primary objects, which is tuned to detect blob-like structures. The
329 "apoptosis" signal is obtained by measuring the maximum intensity over each
330 nucleus object of the "Green" fluorescence image. A threshold value for this
331 intensity I_t is chosen, which defines the fluorescence intensity considered as
332 sufficient for a given nucleus to be considered as apoptotic. In the results
333 presented herein, the threshold chosen was 0.2 that corresponds to 2/3 to 1/2
334 of the maximum intensity of the Green signal.

335 ***Statistical test***

336 For a given time frame, segmentation data consist in a sequence of nuclei
337 positions (X_i, Y_i) and green signal intensity I_i , with i varying from 1 to N , where
338 N is the total number of nuclei objects for this time frame.

339 ***Apoptotic variable A***

340 Given the intensity threshold I_t , we defined the class A of apoptotic cells as
341 the set of nuclei such that $I_i \geq I_t$.

342 ***Clustering (density) C and apoptotic clustering C_a variables***

343 In order to separate "clustered" cells from isolated ones, we set a
344 characteristic interaction distance δ and a threshold n on the number of
345 neighbors. An object at position (X_i, Y_i) is considered to be part of a cluster
346 (*i.e.*, in the class C) if there are a least n nuclei inside the disk of radius δ and
347 center (X_i, Y_i) . An object at position (X_i, Y_i) is considered to be part of an
348 apoptotic cluster (*i.e.*, in the class C_a) if there are a least n apoptotic nuclei
349 (*i.e.*, in the class A) inside the disk of radius δ and center (X_i, Y_i) .

350 ***Independence tests***

351 We used classical Fisher's exact test to determine if random variables A and
352 C are independent and subsequently if random variables A and C_a are
353 independent. In this setting, for the simplicity of notation, we identify the class
354 A with the random variable that indicates if an object belongs or not to the
355 class A, and similarly for classes C and C_a . We gave exact p -values for this
356 test. Generally, the null hypothesis (the considered pair of random variables

357 are independent of each other) can be rejected when the p -value is lower than
358 0.05.

359

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366 **Author contributions**

367 Conceptualization, G. Ichim, D. Coulette, H.L. and E.G.; Methodology, G.
368 Ichim, D. Coulette, H.L. and E.G.; Formal analysis, G. Ichim, D. Coulette and
369 H.L.; Investigation, G. Ichim, D.C., D. Coulette and H.L.; Resources, G. Ichim,
370 D. Coulette, H.L. and E.G.; Writing – Original Draft and Editing, G. Ichim; All
371 authors reviewed and edited the manuscript; Supervision, G. Ichim, D.
372 Coulette, H.L. and E.G.; Project administration and funding acquisition, G.
373 Ichim.

374 **Figure legends**

375 **Figure 1. Imaging and quantification of apoptosis induction using live**
376 **and dead cell markers.**

377 **A.** Overview of the live/dead cell imaging and computer analysis protocol.

378 **B.** Apoptotic signaling induced by treatment with TNF α /CHX or BH3 mimetics,
379 or through BAX overexpression (aCasp8 - active Caspase-8; aCasp 3/7 –
380 active Caspase-3 and -7, tBID – truncated BID).

381

382 **Figure 2. Validation of the apoptosis induction protocols.**

383 **A.** WM115 H2B-mCherry cells were treated either with TNF α /CHX (TC) (50
384 ng/mL of TNF α and 5 μ g/mL CHX) or BH3 mimetics ABT-737/UMI-77 (10 μ M
385 each) for 12 hours in the presence or absence of the pan-caspase inhibitor Q-

386 VD-OPh (10 μ M). Protein lysates were then probed for the expression and
387 processing of PARP1 and Caspase-3. Tubulin was used as a loading control.

388 **B.** WM115 H2B-mCherry cells were treated with 1 or 2.5 μ g/mL of doxycycline
389 (dox) to induce BAX expression for 12 hours in the presence or absence of Q-
390 VD-OPh (10 μ M) and then analyzed as described in (A).

391 **C-D.** IncuCyte imager-based quantification of apoptotic WM115 H2B mCherry
392 cells (SYTOX Green-positive cells) following treatment with TNF α /CHX (TC),
393 ABT-737/UMI-77 (**C**) or doxycycline (at 1 or 2.5 μ g/mL) (**D**).

394 **E.** Representative images of phase-contrast, H2B-mCherry and SYTOX
395 Green signal from WM115 H2B-mCherry cells treated as described in (A).

396

397 **Figure 3. Apoptosis occurs independently of cell clustering.**

398 **A-B.** Definition of the parameters taken into account for the mathematical
399 analysis of the effect cell clustering has on apoptosis induction. Cells with the
400 nucleus half in red half in green are either alive or apoptotic, respectively.

401 **C-E.** Probability estimates (upper panels) and the corresponding *p-value*
402 (lower panels) for the occurrence of apoptosis in TNF α /CHX-treated WM115
403 H2B-mCherry cells surrounded by 1, 2 or 3 neighboring cells.

404 **F-G.** Similar analysis as in (C-E), for WM115 H2B-mCherry cells treated with
405 ABT-737/UMI-77 (**F**) and doxycycline (**G**). The neighboring effect for 1 cell is
406 shown.

407

408 **Figure 4. Apoptotic cell clusters influence the life-death decisions in**
409 **neighboring cells.**

410 **A-C.** Probability estimates (upper panels) and the corresponding *p-value*
411 (lower panels) for the occurrence of apoptosis in TNF α /CHX-treated WM115
412 H2B-mCherry cells surrounded by 1, 2 or 3 apoptotic neighboring cells.

413 **D-E.** Similar analysis as in (A-C), for WM115 H2B-mCherry cells treated with
414 ABT-737/UMI-77 (**D**) and doxycycline (**E**). The neighboring effect for 1
415 apoptotic cell is shown.

416

417 **Supplementary Figure 1 (related to Figure 3).**

418 **A, B.** Probability estimates (upper panels) and the corresponding p -value
 419 (lower panels) for the occurrence of apoptosis in ABT-737/UMI-77-treated
 420 WM115 H2B-mCherry cells surrounded by 2 or 3 neighboring cells.

421 **C, D.** Similar analysis as in (A, B), albeit apoptosis was triggered by
 422 doxycycline induction of pro-apoptotic BAX protein.

423

424 **Supplementary Figure 2 (related to Figure 4).**

425 **A, B.** Probability estimates (upper panels) and the corresponding p -value
 426 (lower panels) for the occurrence of apoptosis in ABT-737/UMI-77-treated
 427 WM115 H2B-mCherry cells surrounded by 2 or 3 apoptotic neighboring cells.

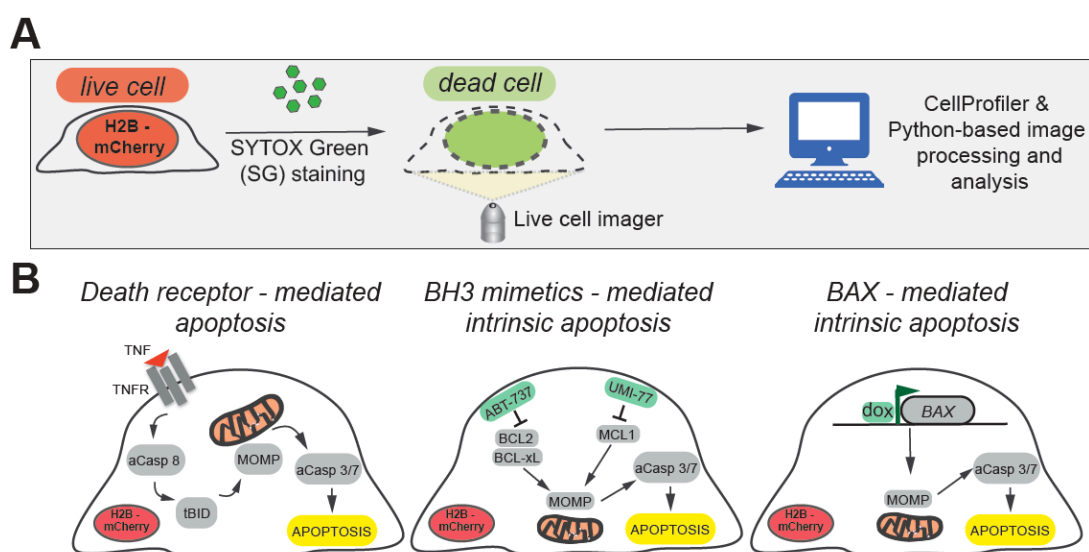
428 **C, D.** Similar analysis as in (A, B), albeit apoptosis was triggered by
 429 doxycycline induction of pro-apoptotic BAX protein.

430

431

432

Figure 1



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Figure 2

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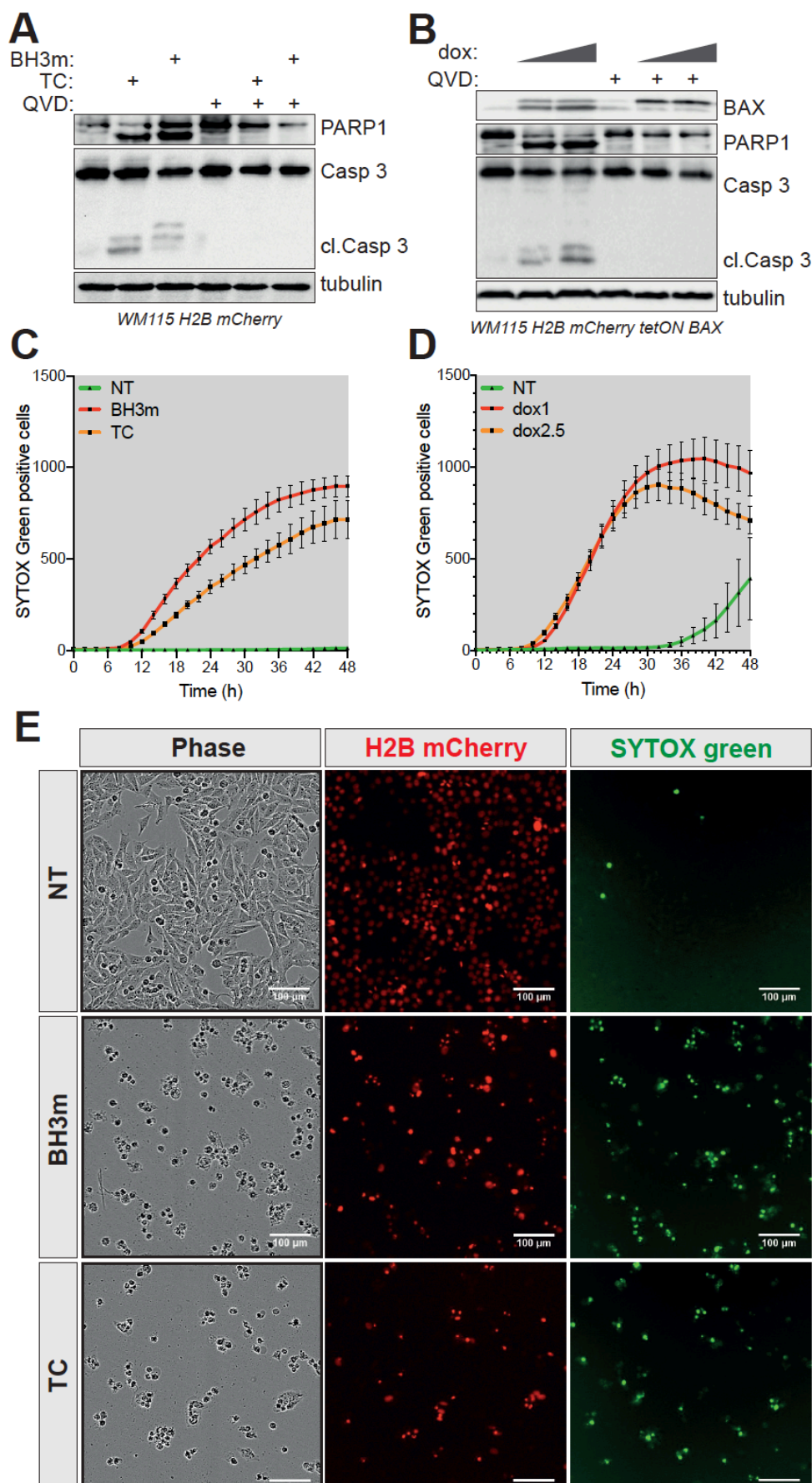
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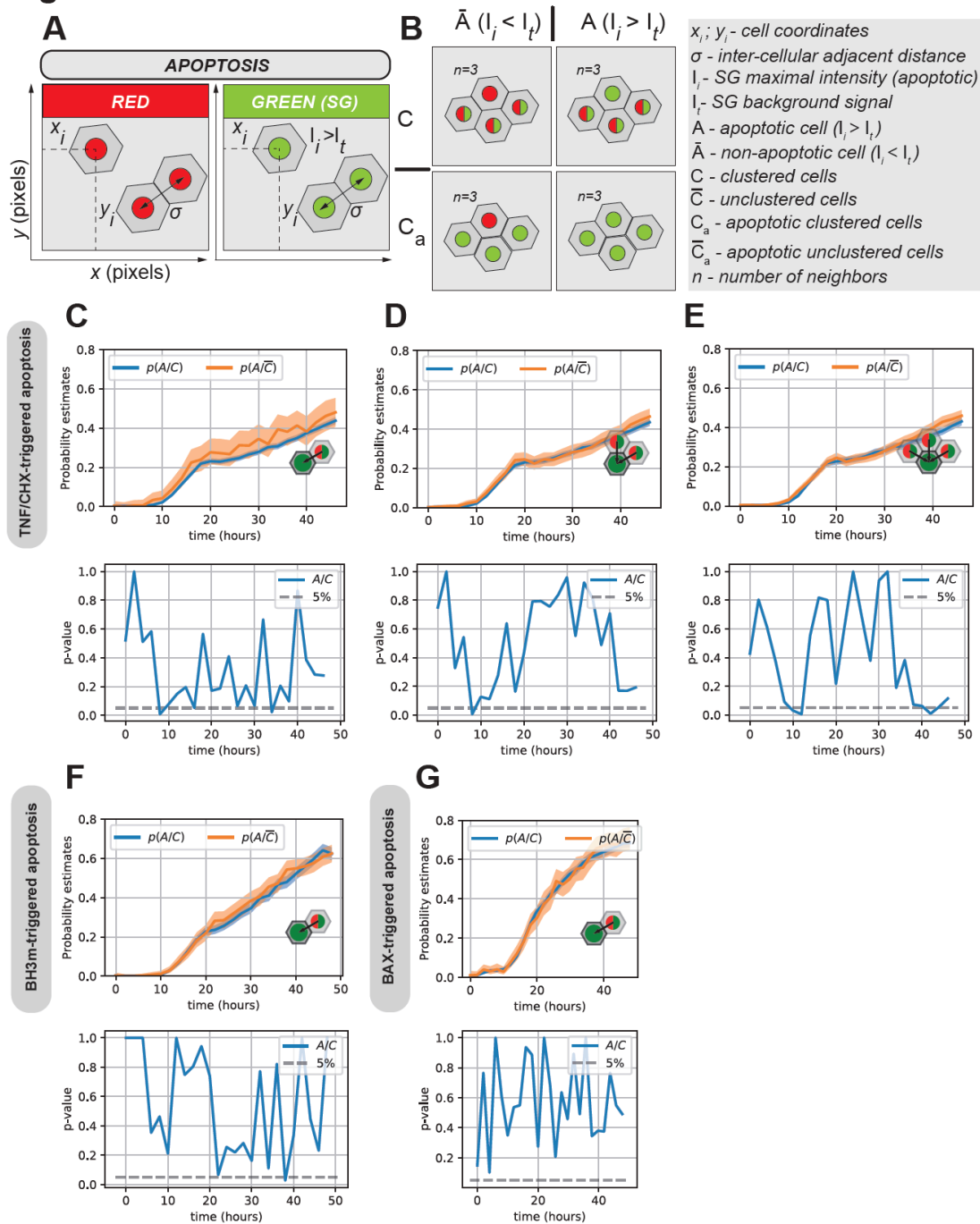
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Figure 3



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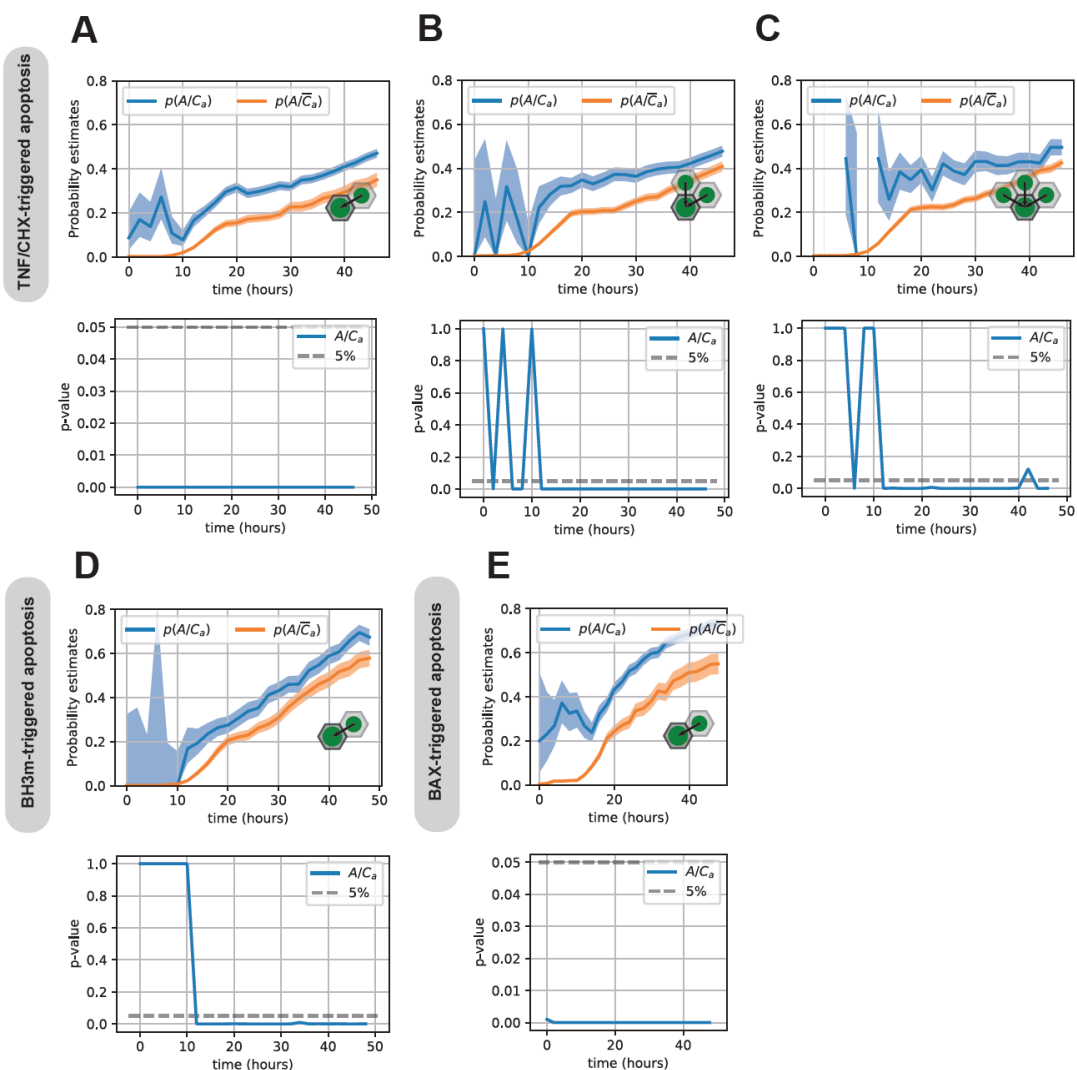
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Figure 4



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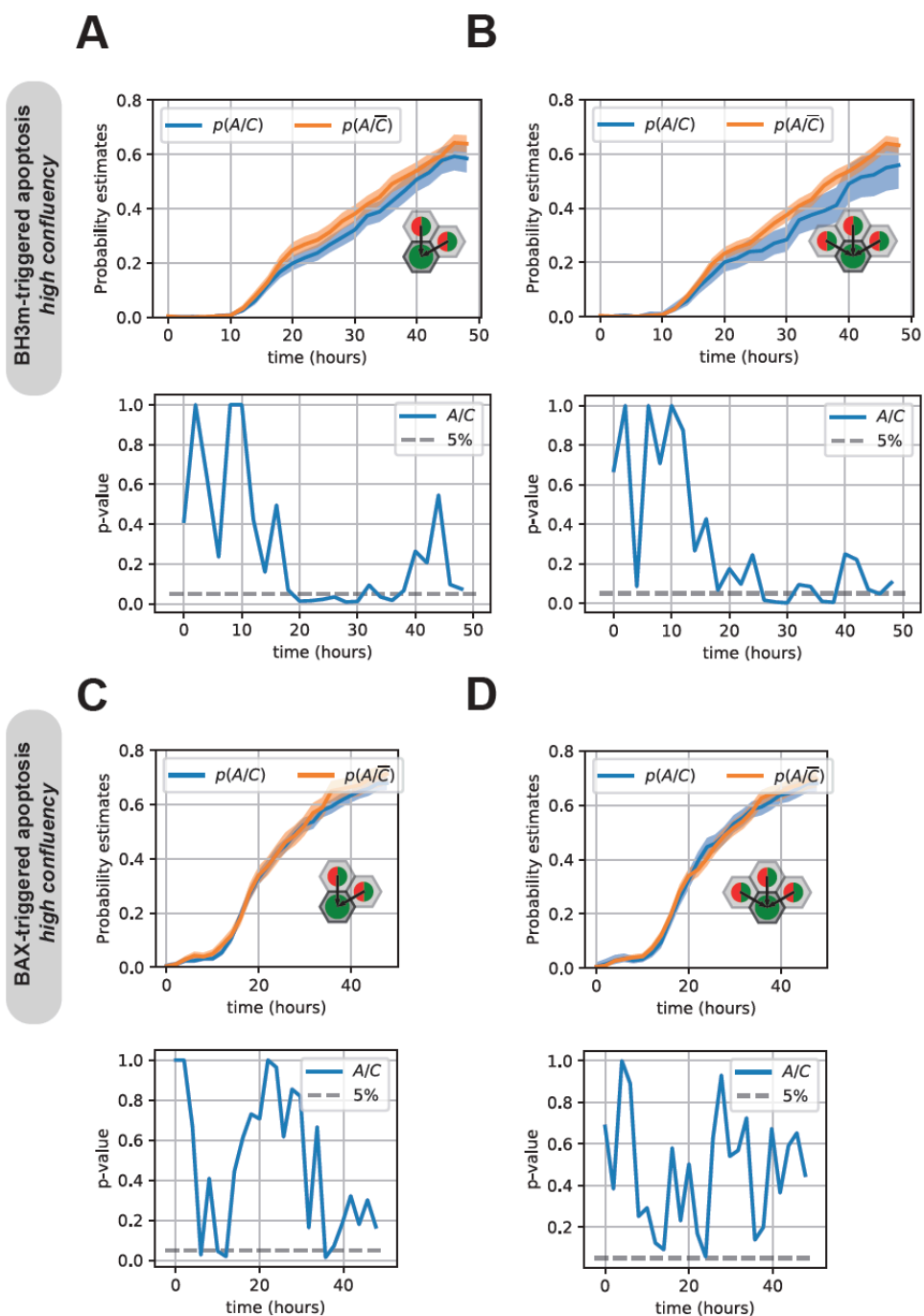
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Supplementary Figure 1



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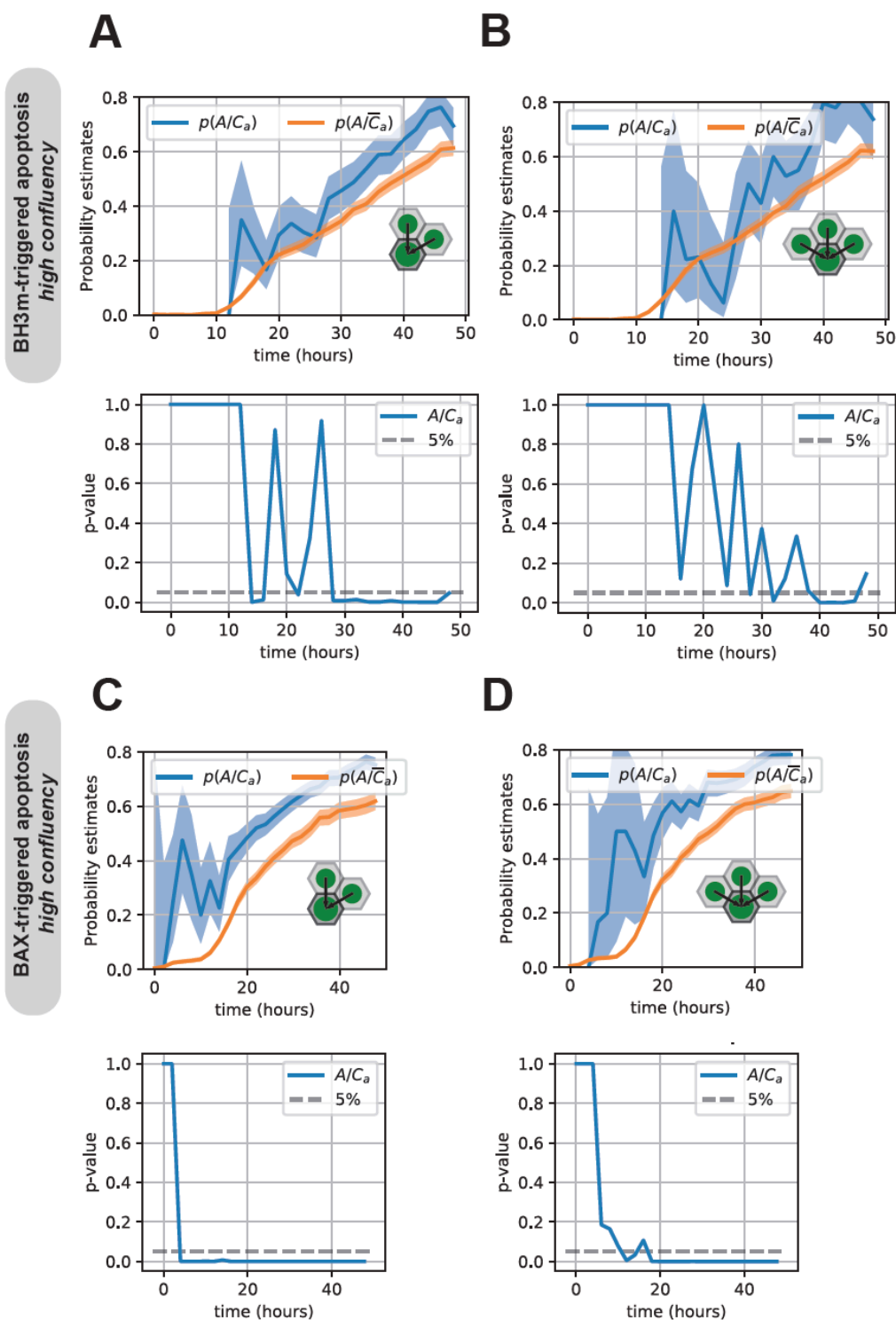
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Supplementary Figure 2



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