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Automated Analysis of Invadopodia Dynamics in Live Cells

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Abbreviations: DMSO; Dimethyl Sulfoxide, ECM; extracellular matrix, FAK; Focal Adhesion Kinase, PBS; Phosphate Buffered Saline, MMPs; matrix metalloproteinases

34 **Abstract**

35 Multiple cell types form specialized protein complexes, podosomes or invadopodia and
36 collectively referred to as invadosomes, which are used by the cell to actively degrade the
37 surrounding extracellular matrix. Due to their potential importance in both healthy physiology as
38 well as in pathological conditions such as cancer, the characterization of these structures has
39 been of increasing interest. Following early descriptions of invadopodia, assays were developed
40 which labelled the matrix underneath metastatic cancer cells allowing for the assessment of
41 invadopodia activity in motile cells. However, characterization of invadopodia using these
42 methods has traditionally been done manually with time-consuming and potentially biased
43 quantification methods, limiting the number of experiments and the quantity of data that can be
44 analysed. We have developed a system to automate the segmentation, tracking and quantification
45 of invadopodia in time-lapse fluorescence image sets at both the single invadopodia level and
46 whole cell level. We rigorously tested the ability of the method to detect changes in invadopodia
47 formation and dynamics through the use of well-characterized small molecule inhibitors, with
48 known effects on invadopodia. Our results demonstrate the ability of this analysis method to
49 quantify changes in invadopodia formation from live cell imaging data in a high throughput,
50 automated manner.

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65 **Introduction**

66 Migration through a three dimensional environment, such as during embryonic development or
67 metastasis, is a multistage process beginning with the migration of either single cells or groups of
68 cells away from the primary site and into the surrounding ECM. To accomplish this migration,
69 the ECM is commonly degraded, typically through the use of matrix metalloproteinases (MMPs)
70 to form paths through which the cells can move. Invadopodia and podosomes, collectively
71 termed invadosomes, are the two structures most commonly associated with this behavior. In the
72 case of cancer cells and the process of metastasis, the formation of invadopodia that actively
73 degrade the ECM is a common observation (Destaing et al., 2011; Murphy and Courtneidge,
74 2011). Importantly, while markers such as cortactin and Arp2/3 subunits can help discern these
75 structures from other actin-rich structures, the direct observation of degradation activity is
76 required in order to accurately identify these invasive structures.

77
78 Invadopodia were first imaged in Rous sarcoma virus transformed cells using several imaging
79 methodologies including interference reflection and fixed cell labelling (Chen, 1989; Tarone et
80 al., 1985). To measure degradation activity *in vitro*, quantitative imaging assays of invadopodia
81 behaviour have been developed that use fluorescently labeled ECM to visualize regions of
82 degradation caused by invadopodia (Artym et al., 2009). By combining fluorescently labeled
83 ECM and fluorescently labelled intracellular markers of invadopodia, such as cortactin (Artym et
84 al., 2006), cofilin (Stoletov et al., 2013) or actin (Albiges-Rizo et al., 2009), the activity of single
85 invadopodia can be followed through time. The continued development of methods to observe
86 invadopodia formation and ECM degradation has made it possible to quantify the effect of
87 siRNA knockdown or drug treatment on invadopodia dynamics. However, the time-lapse image
88 sets produced using these methods have traditionally been analysed using manual analysis
89 methods which are time-consuming and potentially biased in the selection of which invadopodia
90 will be measured.

91
92 In order to improve the reliability of invadopodia measurement systems, we have developed a
93 system to automate the segmentation, tracking and quantification of invadopodia in time-lapse
94 fluorescence image sets at both the single invadopodia level and whole cell level. These methods
95 use the fluorescently labeled ECM images to determine when and where individual invadopodia

96 or cells are degrading the matrix, making detailed studies of invadopodia formation, timing and
97 activity possible. Since these methods were initially developed using LifeAct-GFP as marker of
98 invadopodia, the system does not assume that all bright puncta will become invadopodia and
99 degrade the ECM and instead uses the changes in the underlying ECM to classify puncta. We
100 have made these tools available as open source packages and made a web-based version of the
101 single invadopodia analysis available.

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105 **Results**

106 **Identification and Tracking of LifeAct-GFP Puncta**

107 The development of software and subsequent analysis of invadopodia was performed in multiple
108 steps. The first step of analysis involved the development of methods to segment and track single
109 actin aggregates, termed puncta, from images of LifeAct-GFP. After identifying actin puncta, a
110 set of measurements were made from the fluorescent ECM images at each time point to classify
111 the effect of the puncta on the underlying ECM. As only a portion of the population of LifeAct-
112 GFP puncta degrade the matrix, puncta that degrade the matrix are classified as invadopodia.
113 Additional collected properties such as degradation rates were then used in further analyses as
114 described below.

115

116 To identify LifeAct-GFP labelled puncta in WM2664 cells (Figure 1A), untreated and BB94
117 treated cells were imaged by time-lapse microscopy. In order to ensure that the automated
118 system would have decision thresholds similar to that of experts doing a manual analysis, prior to
119 automated analysis, images were manually segmented to identify LifeAct-positive puncta by
120 three independent observers. A consensus of segmentation was reached by majority vote from
121 the manual segmentation, which was then used to test potential segmentation strategies and
122 determine appropriate filters.

123

124 The first stage of the automated segmentation pipeline used a high-pass filter to remove the
125 background noise from the LifeAct-GFP signal (Figure 1B) and then determined the mean and
126 standard deviation of the high-pass filtered pixel intensities for use as thresholds (Haier et al.,

127 2003). To identify individual puncta, a seed-based region-growing segmentation method was
128 used. To identify seed pixels in each image, intensity thresholds from 1-10 standard deviations
129 were tested and these automatically segmented regions were compared to those identified
130 through manual segmentation (Figure 1C). The false positive and negative rates were computed
131 as previously described (Matov et al., 2010). As the seed threshold increased, the rate of false
132 positives decreased, while the rate of false negatives increased. We also tested minimum seed
133 sizes, ranging from 2-10 pixels, and observed the same general behaviour in the false positive
134 and false negative rates as seed size increased. To balance these factors, we empirically selected
135 a standard deviation threshold of 3 and a minimum size of 6 pixels to identify the puncta seeds
136 (Figure 1D).

137
138 After identification of the puncta seeds, a second threshold was selected to expand around each
139 of the identified seed regions. To assess the performance of the seed expansion procedure, we
140 measured the degree of overlap between the manually segmented puncta and the matching
141 computer segmented puncta. We tested thresholds from 0-3 standard deviations from the mean
142 (Figure 1E). As expected, as the seed expansion threshold increases, the false positive rate
143 decreases, while the false negative rate increases. We selected a seed expansion threshold of 1.75
144 to balance these two factors. As a final filter, we also considered the area and ratio between the
145 major and minor axes of the segmented puncta. To determine these filters, we measured these
146 properties in the manually identified puncta (Figure 1F). We used the minimum and maximum
147 values for the area and the major over minus axes ratio as filters for any objects identified after
148 seed identification and expansion. The cell edge was also identified in the LifeAct-GFP signal
149 using a previously published method (Hoshino et al., 2013). The properties for each identified
150 puncta (Figure 1G) were then collected, which included area and the distance of the puncta from
151 the nearest cell edge.

152
153 Each puncta was also tracked through the experiment, using overlap in adjacent frames to
154 connect the segmented puncta. The majority of the identified puncta were present for only one
155 frame (Figure 2A), but a population of puncta that could be followed for 12 frames or more
156 (Figure 2A inset) was also observed. For puncta that live for 12 frames or more, the average area

157 (Figure 2B) and the average distance from the nearest cell edge (Figure 2C) were calculated over
158 time.

159

160 **Determination of ECM Degradation by Puncta**

161 As described previously, degradation of the matrix is a necessary condition for an individual
162 puncta to be classified as a functional invadopodia. This degradation is visible as dark regions
163 that develop in a fluorescently labelled gelatin matrix underneath puncta over time. In order to
164 detect this change in the ECM, the average ECM intensity immediately underneath each puncta
165 and the area in a five pixel border surrounding the puncta was tracked over time (Figure 3A).
166 Areas within the surrounding border occupied by another identified puncta were excluded from
167 quantitation. The difference between the average intensity in the surrounding ECM and the ECM
168 underneath each puncta was used to calculate the local fluorescence difference, so that puncta
169 that have degraded the ECM will have positive values in the local fluorescence difference, while
170 non-degrading puncta will have values near zero. To account for irregularities in the gelatin
171 matrix, the intensity of the matrix before the puncta appeared in the time-lapse image set was
172 also determined. This pre-birth local fluorescence difference was calculated over the same pixels
173 using the image immediately before the appearance of puncta. In cases where the puncta is
174 present at the beginning of the time-lapse, the first image of the ECM time-lapse was used. The
175 pre-birth local difference calculated at each image was used to correct the observed local
176 intensity difference, giving the corrected local intensity difference.

177

178 To classify the puncta as active invadopodia, the values of the local difference, pre-birth local
179 difference and the corrected local difference were analysed. To ensure that sufficient data from
180 each puncta was assessed, we limited our search to only those puncta present in the time-lapses
181 for at least one hour (12 images under our experimental protocol). Application of this filtering
182 constraint left 2,323 untreated puncta and 979 DMSO, 294 BB94, 533 Focal Adhesion Kinase
183 (FAK) inhibitor, 83 PP2 and 125 Purvalanol A treated puncta. For invadopodia, we would expect
184 the local difference and corrected local difference values to be positive (puncta that do not
185 degrade the matrix; see a sample invadopodia in Figure 3B) and for both of these values to
186 average around zero or negative for non-invadopodia puncta (see a sample non-invadopodia
187 puncta in Figure 3C). Next, we tested whether the mean local intensity difference and the mean

188 corrected local intensity difference were statistically different from zero using a t-test. After
189 applying a bonferroni correction for the number of tests run, 336 untreated, 44 control, 12 BB94
190 treated and 35 FAK inhibitor treated puncta were classified as invadopodia. As expected, no
191 actively degrading invadopodia were detected in the PP2 or Purvalanol A treated cells. The mean
192 local corrected difference was greater in the untreated, control and FAK inhibitor treated cells as
193 compared to the BB94 treated cells (Figure 3D). The calculated differences in invadopodia
194 number following drug treatment correlate with previously identified outcomes on invadopodia
195 for each inhibitor tested (Chan et al., 2009; Hoshino et al., 2012; Wang et al., 1994) and
196 demonstrate the accuracy of the automated detection methods developed here. The 7
197 invadopodia identified in BB94 treated cells were manually identified as false positives and we
198 excluded them from further analysis. The low number of false positives identified in the BB94
199 treated cells further demonstrates the improved performance capable through the use of these
200 automated imaging methods.

201

202 **Measurement of Invadopodia Properties**

203 Invadopodia identified in the untreated, control and FAK treated invadopodia were used to
204 measure the mean area of each invadopodia and average distance of invadopodia to the nearest
205 cell edge (Figure 4A, B). No differences were detected in the average area of invadopodia
206 between conditions (Figure 4A). The average distance of invadopodia from the edge of the cell
207 was decreased by 28% in control cells compared to untreated cells and by 38% after treatment
208 with FAK inhibitor (Figure 4B). There was no difference observed in invadopodia distance from
209 the edge between control cells and FAK inhibitor treated cells (Figure 4B). The lifetime of
210 invadopodia showed increases of 45% as compared to the untreated cells following treatment
211 with the FAK inhibitor (Figure 4C).

212

213 The amount of time from puncta formation until maximum degradation was also quantified. To
214 measure this property a smoothed curve was fit to the degradation curve of each invadopodia
215 (e.g. the curve shown in Figure 3B). The earliest time point at which the smoothed values hit
216 90% of the maximum was chosen as the time to maximum degradation. The time to reach
217 maximum degradation was increased by 77% in the FAK treated cells when compared with the
218 untreated cells (Figure 4D).

219

220 **Quantification of Whole-Cell Degradation Behaviors**

221 To complement the single invadopodia analysis, a system for quantifying the ECM degradation
222 capacity of entire cell populations was also developed. As with the individual invadopodia work,
223 WM2664 cells expressing LifeAct-GFP and exposed to the same drug treatments used for single
224 cell analysis were used to test the capabilities of this system. After image pre-processing, cells in
225 the LifeAct-GFP images (Figure 5A) were identified using the same algorithm as that used to
226 find the cell outline in the single invadopodia analysis (Figure 5B). To minimize the number of
227 cell clusters found and make sure results describe measurements for individual cells, we
228 quantified the areas of all observed single cells and multi-cell clusters (Figure 5C). We
229 empirically set a minimum size threshold of 1,500 pixels ($107 \mu\text{m}^2$) to exclude small debris and a
230 maximum size threshold of 20,000 pixels ($1422 \mu\text{m}^2$) to exclude cell clusters from further
231 analysis.

232

233 By identifying and tracking individual cells through time, we observed a bimodal distribution of
234 cell lifetimes (Figure 5D). Objects present for less than 10 hours were often non-adhered cell
235 bodies or other debris in the field of view and were excluded from further analysis. In parallel
236 with the cell images, images were also taken of the fluorescent ECM (Figure 5E). Using the
237 segmented cells and the intensity of the underlying matrix, we were able to observe both cells
238 that degraded the matrix as well as those that did not (Figure 5F).

239

240 **Determination of Cellular ECM Degradation**

241 The segmented cell and ECM images (Figure 5) were used to classify cells as either degraders or
242 non-degraders. To accomplish this, methods similar to the analysis of single invadopodia were
243 used (Figure 6A). More specifically, for cells identified in each image, the average ECM
244 intensity underneath and within a 40 pixel border around each cell was measured, while
245 excluding any region overlapping with another cell and the corresponding regions in the prior
246 ECM image. The average change in fluorescence intensity from the prior image to current image
247 and in the surrounding 40 pixel border was then calculated. To allow these values to be
248 compared across differing ECM intensity regions, the values were saved as the percentage
249 difference between the ECM intensity underneath each cell and the surrounding region.

250 Therefore, invading cells (i.e. those degrading the matrix) would be expected to have lower ECM
251 intensities immediately underneath the cell. We then collected time-series images for each cell
252 present for at least ten hours (example time-series is shown in Figure 6B).

253
254 In addition to the image-by-image assessment of the percentage of the ECM degraded, the
255 overall percentage of fluorescent ECM degraded by each cell was also assessed (Figure 6C). To
256 find the overall degradation percentage for each cell, the area of influence for each cell was
257 determined by finding the amount of time the cell covered each pixel location in the field of
258 view. Any pixel location covered for at least 2.5 hrs was considered to be in that cell's area of
259 influence. The change in fluorescence intensity from the first image of the ECM time-lapse and
260 the area surrounding the cell was then calculated in the same manner as when calculating the
261 percentage change from image to image.

262
263 Each cell was classified as degrading or non-degrading on a per image basis using the between
264 images and total ECM degradation percentages calculated. As the BB94-treatment is expected to
265 block all activity of the MMPs, degradation percentages found in the BB94 treated cells were
266 used as a negative control. We empirically assessed the potential cut-off values to minimize the
267 number of false positives (ie: the number of BB94-treated cells classified as degraders).

268

269 **Measurement of Matrix Degrading Behaviors**

270 With each cell classified as a degrader or non-degrader, we calculated the percentage of cells in
271 each class at half hour intervals throughout the experiment (Figure 7A). In general, treatment
272 with the FAK inhibitor tended to increase the percentage of degrading cells, while Purvalanol A
273 decreased the percentage of degrading cells. Only eight degrading cells were detected under
274 BB94 treatment and these were manually conformed as false positives and excluded from further
275 analysis. These results are comparable to those observed at the single invadopodia level and
276 again confirm the accuracy and low rate of false positives obtained using the developed
277 automated analysis methods.

278

279 The area of ECM degraded by each cell was also quantified. To find the degraded regions of the
280 matrix, the first and last images of the time-lapse were compared; any region where the intensity

281 had decreased by 20% was marked as degraded. These degraded areas were assigned to each cell
282 according to the previously defined area of influence (Figure 7B). FAK inhibitor significantly
283 increased the area degraded by each cell (62% increase compared to control), while PP2 and
284 Purvalanol A each decreased the average area degraded (61% decrease compared to control).
285 Similarly, the rate of degradation was also quantified by dividing the total area degraded by the
286 cell lifetime (Figure 7C). For this property we observed similar trends to the total area degraded
287 with FAK inhibitor treated cells having a higher rate of degradation (23% increase compared to
288 control), while in the PP2-treated cells and Purvalanol A-treated cells, there was a decrease in the
289 degradation rate (37% and 43% decreases compared to control).

293 Discussion

294 Understanding the process of metastasis is an as yet unresolved issue in cancer biology and
295 whose study has important ramifications in disease management and therapy. As the escape and
296 migration of cancer cells from the primary tumor is preceded by degradation of the ECM,
297 invadopodia may play a highly significant role. However, the quantification of their dynamic
298 behaviours has been relatively limited. The framework presented here provides a reliable
299 approach for the quantitative analysis of invadopodia behavior in both single cells and in cell
300 populations over time. To develop this system, we gathered time-lapse image sets of the
301 WM2664 metastatic cancer cell line expressing LifeAct-GFP (Riedl et al., 2008) forming
302 invadopodia over an Alexa 568-labeled ECM. Since F-actin, as labeled by LifeAct, is not a
303 conclusive marker of invadopodia on its own, we used the images from the labeled ECM to
304 classify each F-actin puncta as either an invadopodia or not based on changes in the ECM
305 intensity over time. After classification of the puncta, our system calculates several dynamic
306 invadopodia properties such as lifetime and the time taken to reach maximum degradation levels.
307 To complement the analysis conducted at the single invadopodia level, we also designed an
308 automated system that follows and quantifies degradation activity at the whole cell level. This
309 approach uses images taken at a lower magnification (20X in our experiments) to gather a
310 representative picture of the degradation behaviour of cellular populations through time. Using
311 this system, we can begin to explore dynamic aspects of cancer cell heterogeneity at the single

312 cell level. For instance, the percentage of cells that have degraded the matrix as well as the rate
313 and total amount of degradation performed by each cell can be quantified. Both of these systems
314 were tested using a set of small molecule inhibitors previously demonstrated to block or enhance
315 invadopodia formation (Chan et al., 2009; Hoshino et al., 2012; Wang et al., 1994). Our results
316 are supported by earlier findings but also significantly extend the amount and degree to which
317 invadopodia and cell degradation behaviors can be quantified, all within an automated image
318 analysis framework.

319

320 Many different pharmaceuticals and their potential effects on invadopodia have been examined
321 in fixed or live cell assays in the past. These include drug treatments such as BB94, Purvalanol
322 A, and PP2 which are hypothesized to inhibit the formation of invadopodia (Chan et al., 2009;
323 Hoshino et al., 2012; Wang et al., 1994). BB94 inhibits MMPs, which are the enzymatic
324 components of invadopodia that degrade the matrix (Wang et al., 1994). The effect of BB94
325 treatment is that formed puncta do not go on to degrade the matrix and are thus not formally
326 classified as invadopodia (observation of degradation is a necessary requirement for
327 classification of puncta as invadopodia) (Linder et al., 2011). Live cell imaging experiments of
328 cancer cells treated with BB94 act as means to set thresholds to minimize the number of false
329 positives detected by the system. Purvalanol A inhibits cyclin-dependent kinase which in turn
330 inhibits the tyrosine kinase Src, and PP2 is a more direct inhibitor of the tyrosine kinase Src and
331 other members of the Src family. Src is known to be involved in invadopodia formation that
332 consequently decreases when Src is inhibited by either Purvalanol A or PP2 (Chan et al., 2009;
333 Hoshino et al., 2012). On the other hand FAK Inhibitor II is hypothesized to enhance the
334 formation of invadopodia. Studies using FAK knockdown have demonstrated an increase in
335 invadopodia suggesting FAK regulates and suppresses invadopodia formation (Chan et al.,
336 2009). Therefore FAK inhibitor II, which decreases FAK autophosphorylation and activation,
337 should enhance invadopodia formation. Automated analysis as presented in the current study has
338 shown the same decrease in invadopodia formation using BB94, PP2 and Purvalanol A, as well
339 as an increase in invadopodia formation following treatment with FAK inhibitor II. Our results
340 clearly demonstrate that the software developed accurately detects known changes in
341 invadopodia formation in response to characterized perturbations.

342

343 A wide range of other fluorescent probes and tools are also capable of being used in the
344 automated analyses described here, such as Tks5 (Tang et al., 2013) or cortactin (Artym et al.,
345 2006). Invadopodia proteins need to be fluorescently tagged and be present at invadopodia
346 during the degradation process in order to be assessed using our system. Many of these
347 alternative tags should, in fact, more reliably mark invadopodia than the LifeAct F-actin label
348 used in this work, making analysis with these markers less likely to result in false positives and
349 decrease the requirement for manual confirmation of potential false positives as required in the
350 BB94 samples in the current study. The cell population analysis system can also be adapted to
351 use alternative cell markers such as dyes or membrane associated fluorescent markers and may
352 be further improved by the addition of a nuclear marker, making it possible to reliably split cell
353 clumps using a watershed segmentation (Malpica et al., 1997). Alternative ECM labeling
354 methodologies such as dye-quenched gelatins, or other ECM substrates such as fibronectin can
355 similarly be utilized. This analysis tool is readily adaptable outside of the field of cancer
356 research, for instance, for the examination of the related invasive structure podosomes, which are
357 found in highly migratory cells such as osteoclasts and macrophages (Albiges-Rizo et al., 2009;
358 Block et al., 2008).

359
360 The software to process the labeled puncta in single cells and in cell populations through time
361 has been released as open source packages available through the Gomez lab github repository
362 (<https://github.com/gomezlab/>). In addition, the single cell analysis of individual puncta can be
363 performed through a web application (<http://ias.bme.unc.edu/>), which does not require the user to
364 download or install any software to process a set of invadopodia images. These two
365 complementary analysis systems allow the quantification of invadopodia behavior at the single
366 invadopodia and single cell levels. Combined with high throughput imaging methodologies, this
367 analysis tool will be highly useful in screening small molecule inhibitors for efficacy in
368 inhibiting invadopodia formation in cancer cells as well as quantifying genetic and cellular
369 heterogeneity that may underlie related metastatic behaviors.

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374 **Materials and Methods**

375 *Reagents*

376 All chemicals and reagents were purchased from Sigma (St Louis, MO) unless otherwise stated.
377 The BB94, Purvalanol A, FAK Inhibitor II and PP2 compounds were all purchased from EMD
378 BioSciences (La Jolla, CA). All drugs were soluble in DMSO, which was used as a control for
379 all experiments. The BB94 and FAK Inhibitor II were used at a final concentration of 5 μ M,
380 while Purvalanol A was used at 2 μ M and PP2 was used at 10 μ M. The Alexa Fluor 568 protein
381 labeling kit used to label the gelatin matrix was purchased from Invitrogen (Eugene, OR), as
382 were all cell culture reagents. Fugene 6 used for transfections was purchased from Roche
383 Diagnostics (Indianapolis, IN). For dynamic visualization of actin, the LifeAct peptide (Riedl et
384 al., 2008) was cloned into the previously described pLL 5.0 GFP Lenti-viral base vector (Wang
385 and McNiven, 2012).

387 *Cell Lines and Culture*

388 Wild Type and LifeAct-GFP WM2664 cell lines and 293FT Hek cells were maintained in
389 DMEM with 10% FBS and 1% PSG at 37°C in a humidified atmosphere with 5% CO₂.
390 Lentiviral production was performed as previously described in (Wang and McNiven, 2012).
391 Briefly, lentiviral expression plasmids were transfected into 293FT cells with the construct of
392 interest using Fugene6. After 24 hrs, media is changed and viral media is applied to 30-50%
393 confluent WM2664 cells in a 6-well dish for 48hrs. Fluorescent cells were FACS sorted for the
394 20-80% range of GFP positive cells.

396 *Fluorescent Gelatin Matrix*

397 To prepare ECM substrate for the invadopodia assays, 0.2% Type A gelatin from porcine skin
398 was fluorescently labeled using the Alexa Fluor 568 protein labeling kit according to the
399 manufacturer instructions. Culture vessels were coated at room temperature with thin layers of
400 20% poly-L-lysine for 20 mins followed by 0.5% glutaraldehyde (Electron Microscopy Sciences,
401 Hatfield, PA) for 15 mins. An 8:1 ratio of unlabeled to labeled gelatin was prepared and
402 incubated on culture vessels for 10 mins. Following gelatin incubation a 5 mg/mL solution of
403 NaBH₄ (Fisher Scientific, Fair Lawn, NJ) was incubated for 15 mins to quench any remaining

404 glutaraldehyde. Between each level of coating, the dishes were washed three times with
405 phosphate buffered saline (PBS).

406

407 *Single Cell Assay Imaging Parameters*

408 LifeAct-GFP WM2664 cells were plated (+/- inhibitors) onto heated biotech dishes (Biotech
409 Inc., Butler, PA) coated with Alexa Fluor 568 labeled gelatin, as described above. Cells were
410 allowed to equilibrate on the microscope 1 hr before imaging. Random fields of view were
411 selected and time-lapse microscopy was performed on an Olympus IX- 81 inverted microscope
412 (60x, 1.3NA objective) with a Hamamatsu CCD camera (model c4742-80-12AG) and a Prior
413 Lumen200Pro epifluorescence system (Olympus America, Center Valley, PA) on a heated stage
414 with heated lid receiving a constant stream of humidified 5% CO₂ to maintain cell viability.
415 Images were captured in both the 488 and 568 channels every 5 mins for 12 hrs using
416 MetaMorph Imaging software (Molecular Devices, Sunnyvale, CA).

417

418 *Cell Population Assay Imaging Parameters*

419 LifeAct-GFP WM2664 cells (+/- inhibitors) were plated onto MatTek 35 mm glass bottom
420 dishes (MatTek Corporation, Ashland, MA) coated with Alexa Fluor 568 labeled gelatin, as
421 described above. Cells were allowed to equilibrate in the incubation chamber of the microscope,
422 heated to 37°C with a humidified atmosphere of 5% CO₂, for 1 hr before imaging. Cells were
423 imaged in the Olympus VivaView microscope with a 20x/0.75NA objective and motorized
424 magnification changer set to 1x, using a Hamamatsu Orca R2 cooled CCD camera (Olympus
425 America, Center Valley, PA). A template was used to select 25 predetermined positions in each
426 dish and images in the 488 and 568 channels were captured every 30 mins for 25 hrs.

427

428 *Invadopodia Analysis*

429 Before analyzing, the fluorescent ECM images were photobleach corrected. To ensure that the
430 degradation of ECM was not incorrectly identified as photobleaching, only pixels outside the cell
431 bodies were considered when photobleaching correction was applied. Images were then flat-field
432 corrected and the single cell time-lapse images for single cell assays were registered (the cell
433 population movies did not require registration). In the ECM images used for the single
434 invadopodia analysis, the average fluorescence outside the cell bodies was set to 1000 to allow

435 the local difference values to be compared between ECM preparations. The LifeAct-GFP images
436 for the single invadopodia analysis were not pre-processed. The LifeAct-GFP images for the
437 population analysis were flat-field corrected. The analysis software uses a high-pass filter and
438 threshold to identify regions of high actin concentration (potential invadopodia) that co-localize
439 with underlying ECM degradation to identify active invadopodia and track them through time.

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541 **Figure Legends**

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543 **Figure 1.** Segmentation of Puncta from LifeAct-GFP images.

544 (A) Epifluorescence image of WM2664 cell expressing LifeAct-GFP. (B) Image from Part A
545 passed through a high-pass filter. (C) Contour plots showing the detection errors for
546 identification of puncta seeds. (D) Locations of puncta seeds accepted with minimum seed size 6
547 and high-passed threshold of 3. (E) Error rates on a pixel basis as a function of the puncta
548 expansion threshold. (F) Puncta area plotted versus ratio between major and minor axes in
549 puncta manually identified in either control or BB94 treated cells. (G) Locations of segmented
550 LifeAct-GFP puncta based on seeding, expansion, area and major over minor axes filtering.

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552

553 **Figure 2.** Properties of Segmented and Tracked Puncta in Control Cells

554 (A) Histogram of the lifetime of the segmented puncta. Inset graph shows the lifetime of puncta
555 with lifetime of 12 frames or more. (B) Histogram of the average puncta area for puncta with
556 lifetimes over 1 hr. (C) Histogram of the distance to the nearest cell edge.

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558

559 **Figure 3.** Measurement of ECM degradation underneath single puncta.

560 (A) Cartoon representation of a single puncta and the corresponding ECM underneath that
561 degrading puncta. (B) Small multiple visualization of a single degrading puncta from a control
562 cell and corresponding ECM intensities. The puncta is outlined in green, while the region
563 classified as the local background is shaded purple. The first column shows the LifeAct and
564 ECM images immediately before puncta formation and the last column shows the same areas
565 immediately after puncta disappearance. (C) Small multiple visualization of a non-degrading
566 puncta. (D) Boxplots of the mean local corrected difference in four of the experimental
567 conditions. The box indicates the 25th and 75th percentiles, while the bold line indicates the
568 median and the whiskers extend to 1.5 times the interquartile range. * indicates $p < 0.05$ by t-test.

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570

571 **Figure 4.** Properties automatically extracted from the identified invadopodia. (A) The average
572 area of invadopodia. (B) The average distance from the cell edge of invadopodia. (C) The
573 lifetime of invadopodia. (D) The average time to maximum matrix degradation.

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575

576 **Figure 5.** Identification of LifeAct-GFP Expressing WM2664 Cells. (A) Sample LifeAct-GFP
577 image from a 25 hr time-lapse experiment after photobleaching and flat-field correction. (B)
578 Same image as in part A, with the segmented cells outlined in yellow. (C) The distribution of
579 object sizes detected based on intensity thresholding. (D) The distribution of the lifetime of
580 objects detected in the control time-lapse image sets. (E) Sample fluorescent ECM image from
581 the same time and field as in part A. (F) The ECM channel in the same field as in part E, at the
582 end of the 25 hr experiment. The two sub-images show the first and last image of two cells
583 outlined in red identified in the field. The lower portion of each sub-image shows the ECM
584 channel immediately underneath each cell.

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586

587 **Figure 6.** Determination of Single Cell Degradation Status. (A) Cartoon representation of two cells
588 of which one cell degrades the matrix (Cell #1) and another non-degrading cell (Cell #2). Also
589 shown are the overlapping areas of the cell location and the results of comparing the first and last
590 images from the image set. (B) Example small multiple tracks of single cells through time and
591 the corresponding measurement of the percent of matrix degraded between each image. The
592 colors outlining each image on the left corresponds to the same color line in the plot on the right
593 (C) Boxplots of the overall percentage of fluorescent ECM removed underneath control, DMSO
594 treated and BB94 treated cell. * indicate $p < 0.05$ via t-test.

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596

597 **Figure 7.** Properties of Degrading Cells (A) Percentage of cells classified as degraders over the
598 25 hrs imaging time frame. Percentages are averages from several experiments (n=5 control, n=2
599 BB94, n=14 DMSO, n=4 PP2, n=3 Purvalanol A and n=4 FAK Inhibitor). (B) The total area and
600 (C) rate of degradation from each cell classified as an invader, * $p < 0.05$ via t-test

FIGURE 1

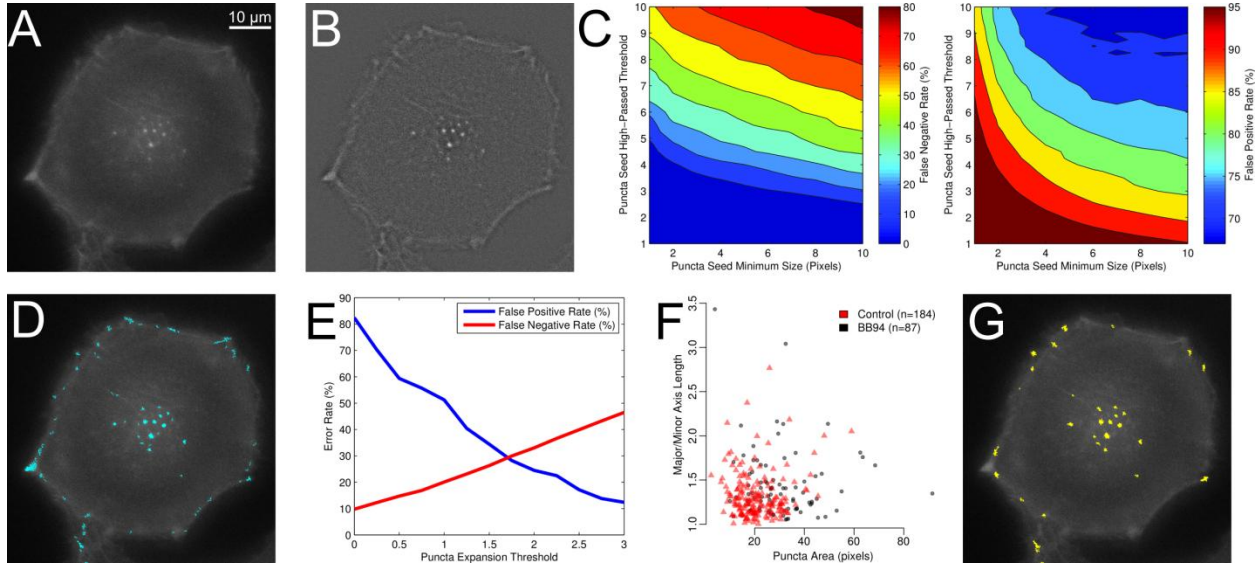


FIGURE 2

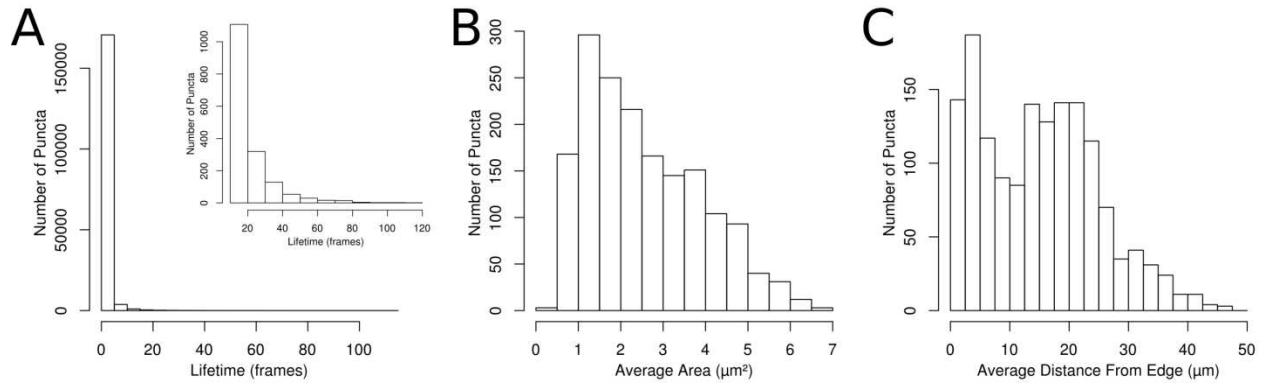


FIGURE 3

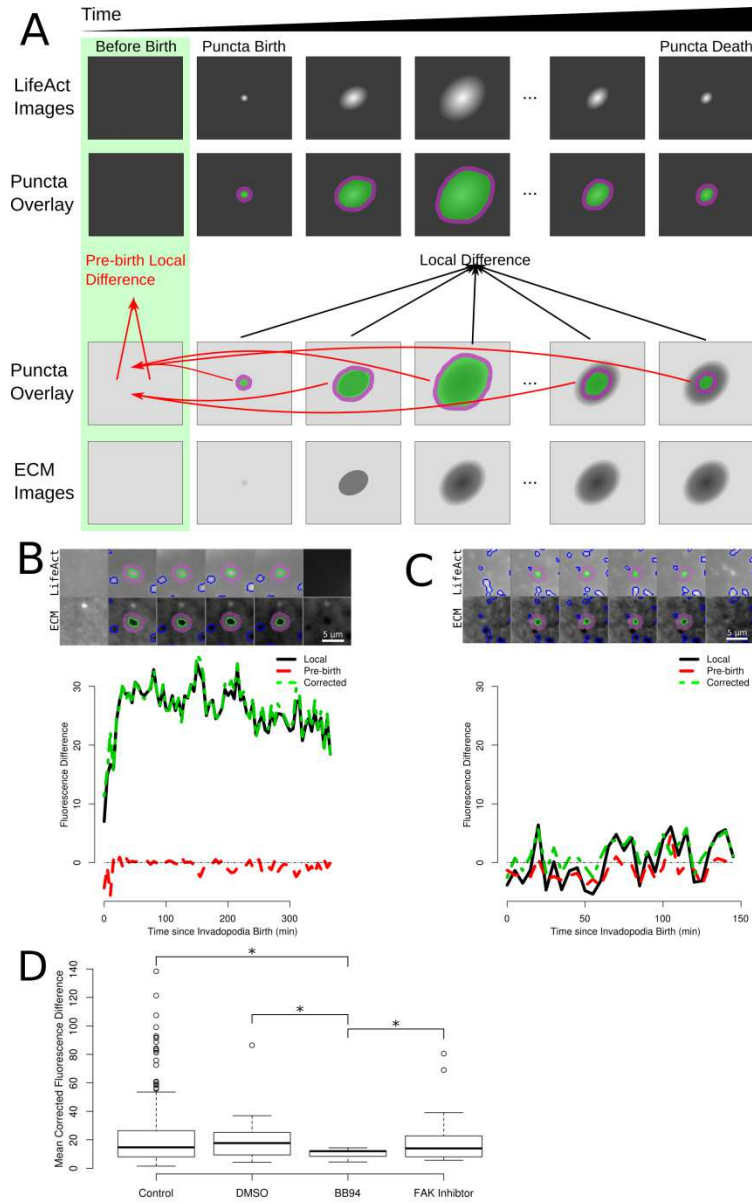


FIGURE 4

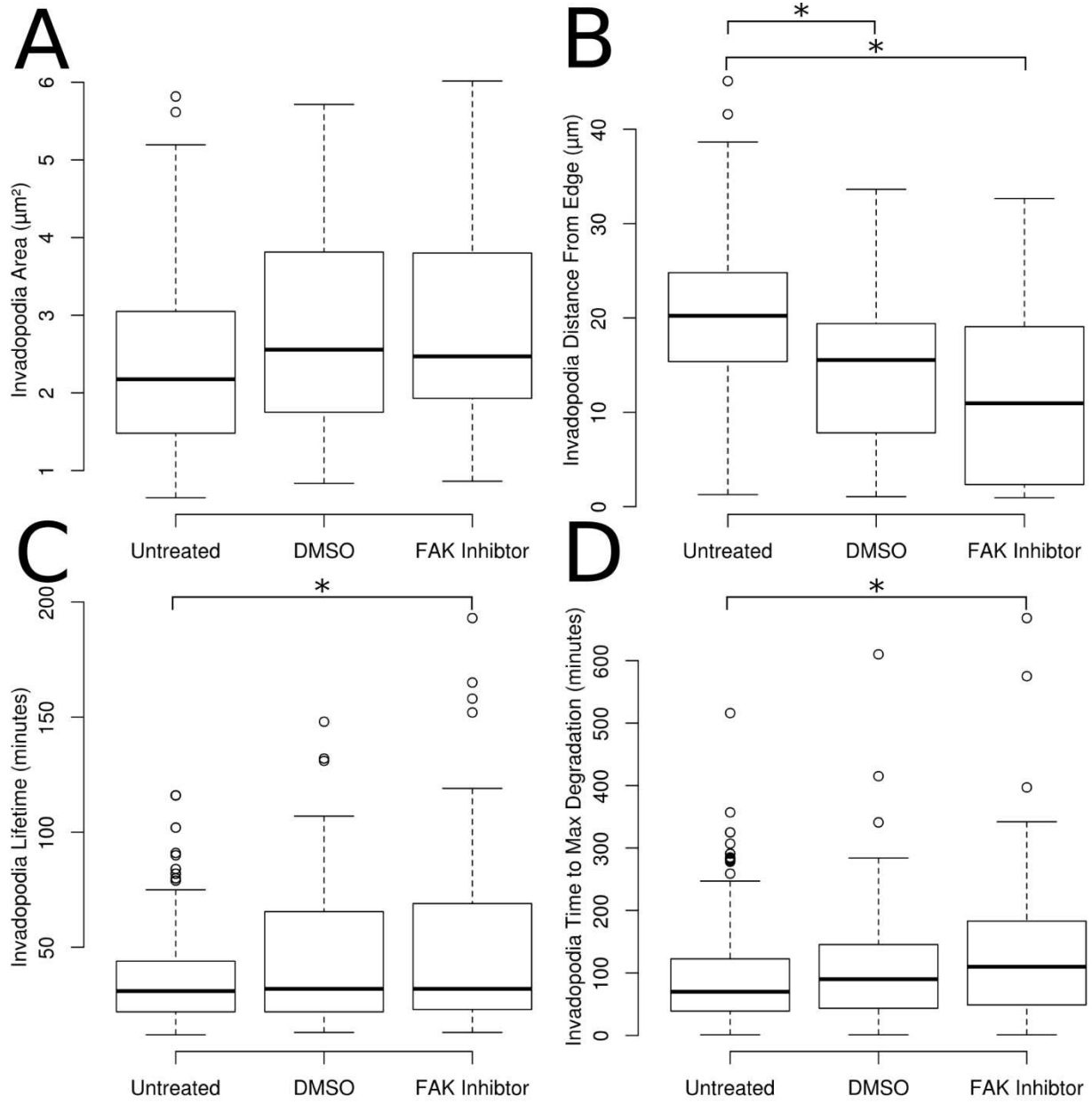


FIGURE 5

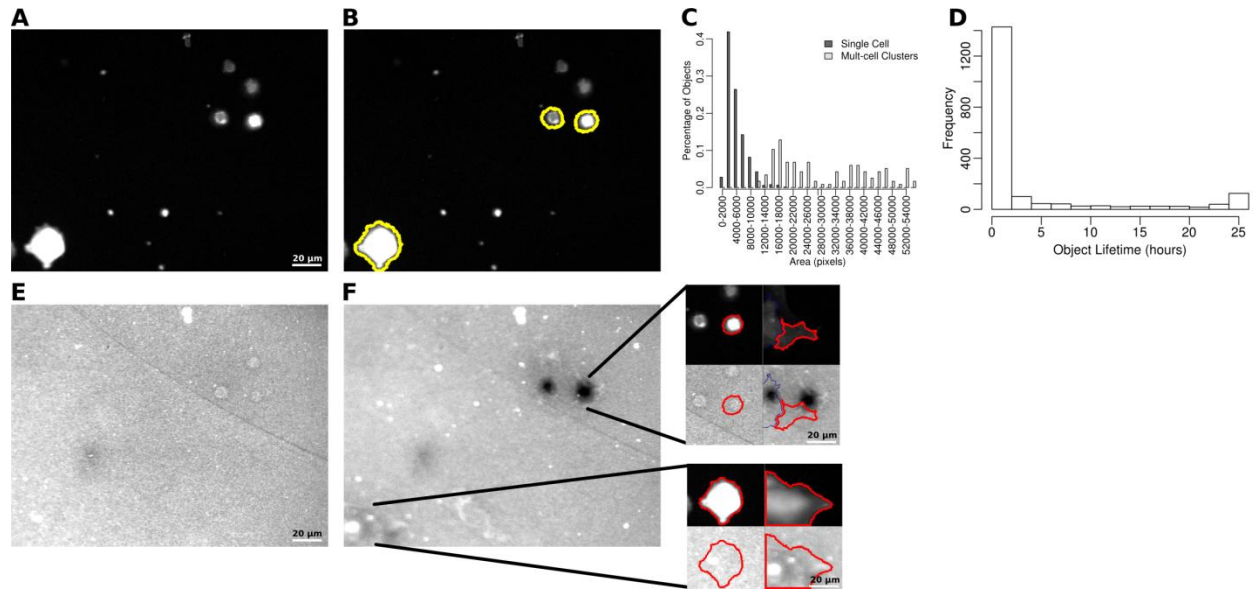


FIGURE 6

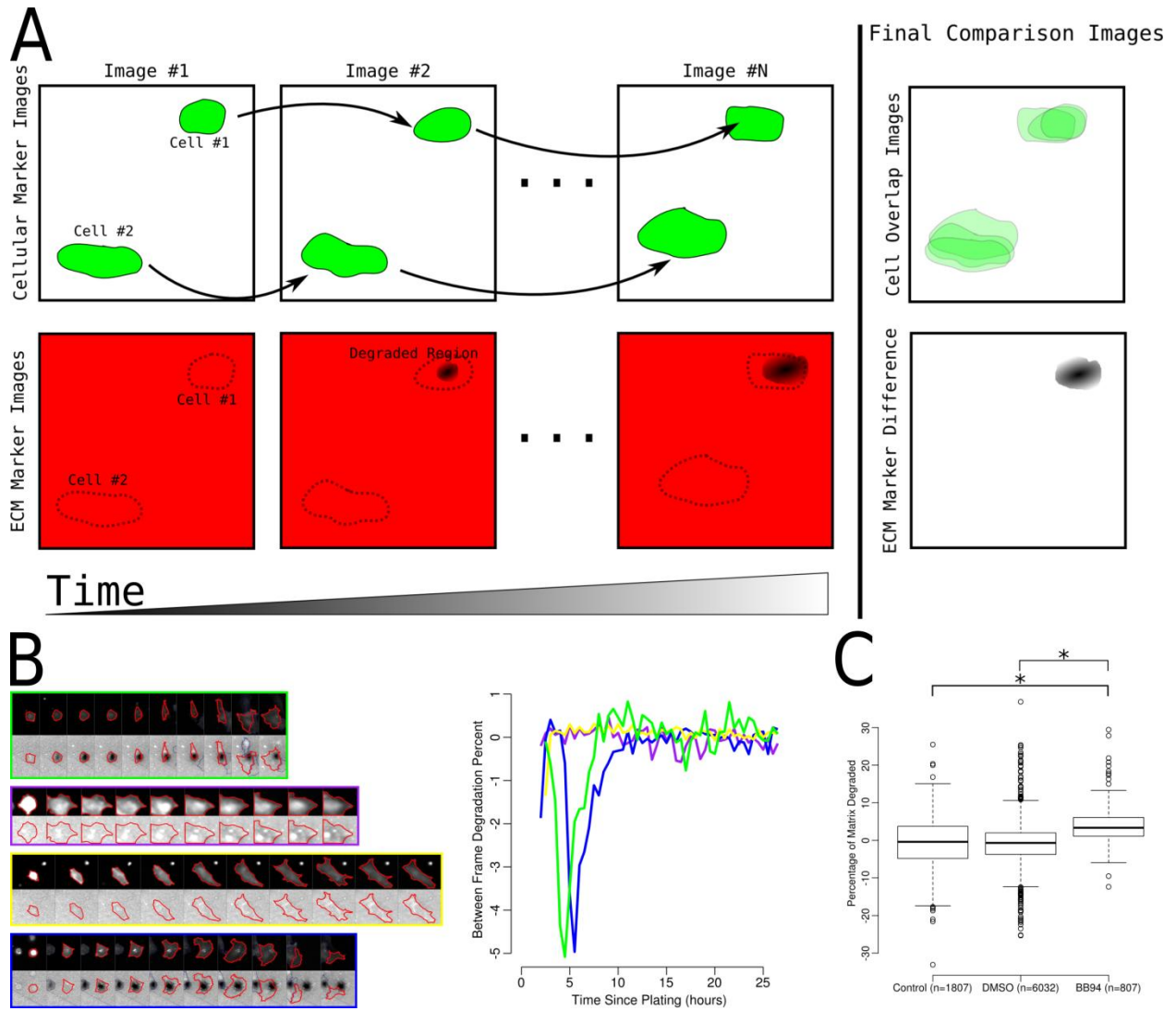


FIGURE 7

