QCM™ Gelatin Invadopodia Assay (Red)

For 4 x 8-Well Chamber Slides

Catalog No. ECM671

FOR RESEARCH USE ONLY

Not for use in diagnostic procedures.

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Introduction

Invasion of cells through layers of extracellular matrix is a key step in tumor metastasis, inflammation, and development. The process of invasion involves several stages, including adhesion to the matrix, degradation of proximal matrix molecules, extension and traction of the cell on the newly revealed matrix, and movement of the cell body through the resulting gap in the matrix (Friedl and Wolf, 2009). Each of these stages of invasion is executed by a suite of proteins, including proteases, integrins, GTPases, kinases, and cytoskeleton-interacting proteins.

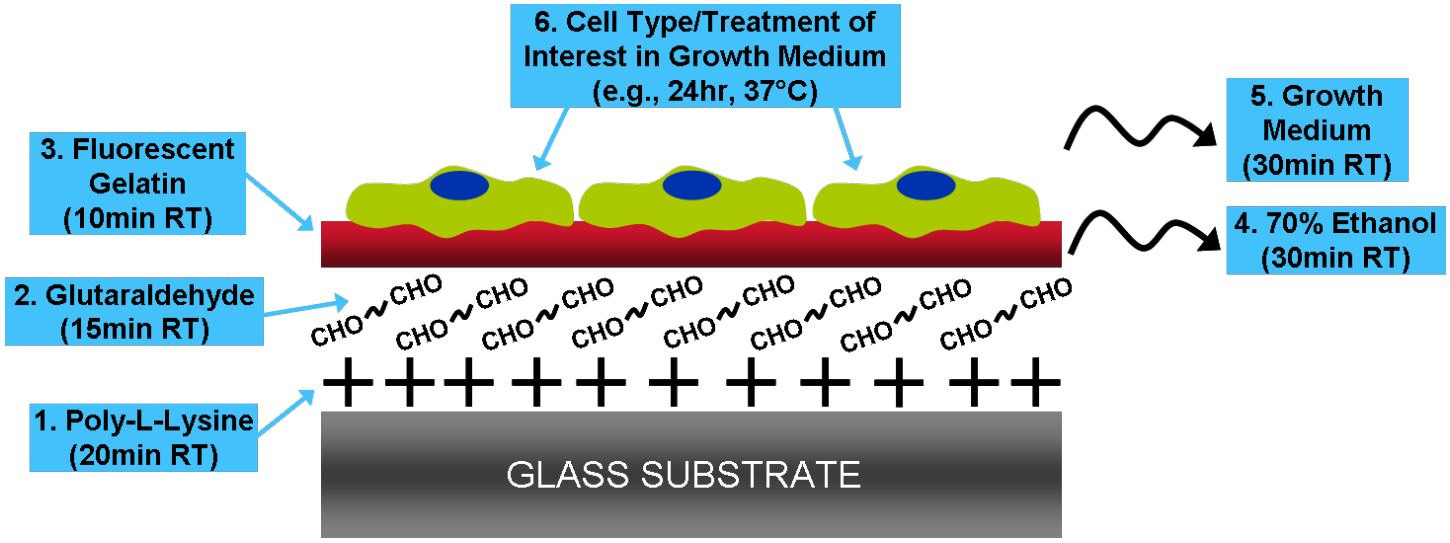
Classical methods for analyzing cellular invasion involve application of cells to one side of a layer of gelled matrix molecules and quantifying the relative number of cells that have traversed the layer. Such methods are extremely useful for analyzing invasion at the cell population level, but analysis of the subcellular events mediating the stages of invasion require techniques with higher resolution. The method that has been most informative for pinpointing regions of the cell that initiate invasion involve plating cells on a culture surface coated with a thin layer of fluorescently labeled matrix, and visualizing regions where the cell has degraded the matrix to create an area devoid of fluorescence (Chen *et al.*, 1984). Such assays have revealed that invasive cells extend small localized protrusions that preferentially degrade the matrix. These protrusions are termed invadopodia in cancerous cells, and podosomes in non-malignant cells such as macrophages (Ayala *et al.*, 2006). Many molecules orchestrate the formation and function of invadopodia; a few of the key molecular events include Src phosphorylation of scaffolding protein Tks5 (Seals *et al.*, 2005), N-WASP activation and cortactin regulation of the Arp2/3 complex to induce actin polymerization (Yamaguchi *et al.*, 2005; Weaver, 2006), and generation of reactive oxygen species by NADPH oxidases (Diaz *et al.*, 2009).

EMD Millipore's QCM™ Gelatin Invadopodia Assays provide optimized materials and protocols to enable reproducible analysis of invadopodia in invasive tumor cells. All of the components necessary for affixing a thin film of fluorescent matrix to glass culture surfaces are provided. In addition, compatible reagents are provided for co-localizing the actin cytoskeleton and nuclei with invadopodial degradation sites. This assay may be used for assessing activity of inhibitors and promoters of invadopodia formation and function. Finally, different cell types as well as individual cells in heterogeneous populations may be analyzed for invasive potential with the QCM™ Gelatin Invadopodia Assay.

Assay Principle

The EMD Millipore QCM™ Gelatin Invadopodia Assay (Red) provides the reagents necessary for affixing a thin, uniform layer of Cy3-labeled gelatin to a glass culture substrate, allowing for rapid detection of matrix degradation (Artym *et al.*, 2009, Xu *et al.*, 2009). A poly-L-lysine coating is first adsorbed to the glass substratum. The substrate is then treated with a dilute glutaraldehyde solution to bi-functionally "activate" the surface for further protein binding. Subsequent incubation of the surface with fluorescent gelatin allows covalent coupling between the poly-L-lysine and gelatin via reactive aldehyde (-CHO) groups. The fluorescently-coated glass is now prepared for cell culture by disinfection with 70% ethanol, followed by quenching of free aldehydes with amino acid-containing growth medium. Upon completion of fluorescent substrate preparation, cell types of interest may be seeded onto the gelatin surface for a desired amount of time. Depending on cell type, degradation may occur within a few to several hours. Treatment compounds of interest may also be introduced within the culture period.

With EMD Millipore's QCM™ Gelatin Invadopodia Assay (Red), degraded areas of gelatin, now devoid of fluorescence, may be microscopically visualized and quantified using image analysis software algorithms. The assay also provides fluorescent FITC-phalloidin and DAPI, for visualization of cytoskeletal F-actin and nuclei, respectively, to allow co-localization of degradation with cellular features. Potential activators or inhibitors of invadopodia formation may be investigated for their influence on the degree and frequency of matrix degradation, and the assay may be further combined with immunocytochemical staining for other molecules of interest in pathway studies.



Application

EMD Millipore's QCM™ Gelatin Invadopodia Assay is a rapid, convenient reagent kit for the generation of thin, uniform fluorescent gelatin layers for use in both invadopodia- and podosome-associated matrix degradation studies. Gelatin degradation may be microscopically imaged during both live- and fixed-cell assays. Each assay kit contains sufficient reagents for the coating of four 8-well glass chamber slides, although alternate substrate formats may also be utilized. The EMD Millipore QCM™ Gelatin Invadopodia Assay is intended for research use only, not for diagnostic or therapeutic applications.

Kit Components

ECM671-1 (2-8°C Storage):

1. Cy3-Gelatin, 4X: (Part No. CS207803) One vial containing 0.5 mL.
2. Unlabeled Gelatin, 4X: (Part No. CS207805) One vial containing 2 mL.
3. DAPI: (Part No. 90229) One vial containing 100 µL at 100 µg/mL in water.
4. Poly-L-Lysine, 2X: (Part No. CS207800) One vial containing 5 mL.

ECM671-2 (-20°C Storage):

5. Glutaraldehyde, 16X: (Part No. CS207801) One vial containing 1 mL.
6. FITC-Phalloidin: (Part No. CS207821) One vial containing 20 µg.

Materials Not Supplied

1. Sterile cell culture hood
2. Pipettors, liquid aspirators, etc. for handling of cells and liquid reagents
3. Sterile plasticware (cell culture flasks, centrifuge tubes, pipettes, pipette tips, etc. for handling of cells and liquid reagents)
4. Sterile glass substrate (e.g., chamber slide, coverslip, glass-bottom dish/multi-well plate)
5. Sterile deionized water
6. Sterile Dulbecco's phosphate-buffered saline (DPBS), without calcium or magnesium
7. 70% ethanol in sterile water
8. Cell type of interest, with appropriate growth medium and cell detachment buffer (e.g., 0.25% trypsin)
9. Hemocytometer (e.g. Scepter™ Handheld Automated Cell Counter)
10. Trypan blue or equivalent viability stain
11. Low speed centrifuge for cell harvesting
12. CO_2 tissue culture incubator
13. 3.7% formaldehyde in DPBS (or equivalent) for cell fixation
14. Methanol for phalloidin reconstitution
15. Blocking/permeabilization buffer for phalloidin/DAPI staining (e.g., 2% blocking serum/0.25% Triton X-100 in DPBS)
16. Slide mounting media (with anti-fade reagent) and cover glasses, if appropriate
17. Microscope/image acquisition system (for phase contrast and fluorescence)
18. Fluorescence filters for Cy3, FITC and DAPI imaging (see **Table 2** for specific excitation/emission wavelengths)
19. Image analysis software (e.g., NIH ImageJ)

Related Products Available from EMD Millipore

PHCC20040	Scepter™ 2.0 Handheld Automated Cell Counter, w/ 40 µM Sensors
PHCC20060	Scepter™ 2.0 Handheld Automated Cell Counter, w/ 60 µM Sensors
BSS-1006-B	EmbryoMax® 1X Dulbecco's Phosphate Buffered Saline
SM-2003-C	0.25% Trypsin-EDTA in Hank's Balanced Salt Solution
05-180	Mouse Anti-Cortactin (p80/85), Clone 4F11
324878	Focal Adhesion Kinase Inhibitor II
CC1100	GM6001 [Ilomastat] MMP Inhibitor
529573	PP2 (Src Inhibitor)
MAB3328	Mouse Anti-MMP-14 [MT1-MMP], Clone LEM-2/15.8
05-184	Mouse Anti-Src, Clone GD11
09-403	Rabbit Anti-Tks5 (SH3 #1)
AB2962	Rabbit Anti-N-WASP
AB3886	Rabbit Anti-Arp2
07-272	Rabbit Anti-Arp3

Precautions

Refer to MSDS for further information.

Component	Hazards
Glutaraldehyde	Toxic, corrosive, sensitizer (handle in fume hood or biosafety cabinet)
Phalloidin	Highly toxic
DAPI	Potential mutagen

Storage

Store Cy3-Gelatin, Unlabeled Gelatin, Poly-L-Lysine and DAPI at 2-8°C. Store Glutaraldehyde and FITC-Phalloidin at -20°C. Use all reagents within 4 months from date of receipt.

Assay Protocol

The given protocol volumes assume preparation of 4 x 8-well glass chamber slides. For alternate per sample volumes, see **Table 1** (or scale volumes as appropriate for culture surface area). All reagents should be diluted to 1X only immediately before use.

Substrate Preparation and Cell Seeding:

Perform the following steps in a sterile cell culture hood with sterile plasticware. Mix all reagents gently before use.

1. Dilute Poly-L-Lysine, 2X solution to 1X with sterile, deionized water by mixing 4 mL Poly-L-Lysine, 2X + 4 mL sterile, deionized water = 8 mL total 1X solution.

2. Add 250 µL of 1X Poly-L-Lysine solution to each well; incubate at room temperature for 20 minutes.

3. Remove poly-L-lysine solution; rinse three times with sterile Dulbecco's PBS (DPBS, without calcium or magnesium) using 500 µL/well.

4. Dilute Glutaraldehyde, 16X solution to 1X with sterile DPBS:
    a. Thaw Glutaraldehyde, 16X to room temperature. Remaining glutaraldehyde may be re-frozen for subsequent use.
    b. Mix 0.5 mL Glutaraldehyde, 16X + 7.5 mL sterile DPBS = 8 mL total 1X solution.

5. Add 250 µL of 1X Glutaraldehyde solution to each well; incubate at room temperature for 15 minutes.

6. Remove glutaraldehyde solution (dispose of as hazardous waste); rinse three times with sterile DPBS using 500 µL/well.

7. Prepare 1:5 mixture of 1X Cy3-Gelatin:1X Unlabeled Gelatin
    a. Dilute Cy3-Gelatin, 4X solution to 1X with sterile DPBS:
        i. Pipet 320 µL Cy3-Gelatin, 4X into a 1.5 mL microcentrifuge tube. Heat at 60°C for 5 minutes.
        ii. Mix 320 µL of heated Cy3-Gelatin, 4X with 960 µL sterile DPBS = 1280 µL total 1X fluorescent solution.
    b. Dilute Unlabeled Gelatin, 4X solution to 1X with sterile DPBS:
        i. Mix 1280 µL Unlabeled Gelatin, 4X + 3840 µL sterile DPBS = 5120 µL total 1X unlabeled solution.
    c. Mix 1280 µL 1X Cy3-Gelatin + 5120 µL 1X Unlabeled Gelatin = 6400 µL total 1X gelatin mixture.

8. Add 200 µL of 1X gelatin mixture to each well; incubate at room temperature for 10 minutes, protected from light.

9. Remove gelatin mixture; rinse three times with sterile DPBS using 500 µL/well.

10. Disinfect substrates with 70% ethanol in sterile water using 500 µL/well; incubate at room temperature for 30 minutes, protected from light.

11. Remove ethanol; rinse three times with sterile DPBS using 500 µL/well.

12. To quench residual free aldehydes, add 500 µL of growth media per well and incubate at room temperature for 30 minutes, protected from light.

13. For cell seeding, use cells that are ~70-80% confluent.
    a. Detach cells as appropriate for cell type of interest.
    b. Remove sample for cell counting (e.g., via hemocytometer, with viability stain).
    c. Centrifuge and resuspend cells in growth media at desired concentration. Seeding densities should be empirically determined for each cell type over desired culture period. Optimal densities may be sub-confluent, to allow for distinction of matrix degradation by individual cells.

14. Remove growth media used for quenching; seed cells in fresh growth media at 500 µL/well. Allow chamber slides to sit undisturbed on a horizontal surface at room temperature for ~15-30 minutes (protected from light) to encourage even cell distribution. Place chamber slides into a 5% CO_2 tissue culture incubator at 37°C for the remainder of the desired culture duration.
    a. Cell treatments may also be added simultaneously with seeding or following an initial adhesion phase (e.g., 2-4 hours).
    b. Total culture and/or treatment time before visualization of gelatin degradation (e.g., 24 hours) should be empirically determined, as appropriate for the cell type/treatment of interest. Note: Degree/time-course of degradation is highly cell type-dependent.

**For coverslip preparation/seeding:

- Individual glass coverslips may also be utilized for gelatin coating.
- A 12 mm-diameter round coverslip may be coated/cultured in a standard 24-well plate.
- Before coating, tracking of coverslip "sides" may be facilitated by labeling the "non-coated side" of each coverslip with a solvent-resistant marker (or other marking strategy).
- Handle coverslips with 70% ethanol-disinfected forceps (serrated tips).
- The poly-L-lysine, glutaraldehyde and gelatin coating steps (with associated DPBS rinses) may be performed by filling the 24-well plate with the recommended reagent volume, then inverting the coverslip onto the liquid ("coated side" down). For alternate coverslip sizes, appropriately-sized dishes or multi-well plates may be utilized, or reagent volumes may be dropped onto Parafilm surfaces, followed by coverslip inversion (volumes may need to be adjusted for size).
- The ethanol, media quench, cell culture, fixation and staining steps (with associated rinses) may be performed by sinking the coverslip in the well ("coated side" up), followed by filling the 24-well plate with the recommended reagent volume. For alternate coverslip sizes, appropriately-sized dishes or multi-well plates should be utilized.

	Glass Substrate (Surface Area per Well or per Sample)		
Per Well or Per Sample 1X Coating/Staining Volumes	8-Well Chamber Slide (0.7 cm^2)	96-Well Plate (0.2 cm^2)	12 mm-Diameter Round Coverslip (1.1 cm^2) in 24-Well Plate (*see **Assay Protocol** for further details)
Poly-L-Lysine	250 µL	70 µL	250 µL (*invert)
DPBS Rinses	500 µL	140 µL	500 µL (*invert/sink)
Glutaraldehyde	250 µL	70 µL	250 µL (*invert)
Gelatin	200 µL	60 µL	200 µL (*invert)
70% Ethanol	500 µL	140 µL	500 µL (*sink)
Growth Media (quench/ cell seeding)	500 µL	140 µL	500 µL (*sink)
Fixative (e.g., 3.7% Formaldehyde)	250 µL	70 µL	250 µL (*sink)
Fluorescent Staining Buffer Rinses (user-provided)	500 µL	140 µL	500 µL (*sink)
Phalloidin/DAPI Staining Solution	200 µL	60 µL	200 µL (*sink)

Table 1. Recommended coating/staining volumes for example glass substrates.

Sample Fixation and Staining:

The following steps do not need to be performed under sterile conditions.

Note: Gelatin degradation may be visualized in live-cell assays, however, phalloidin and DAPI counter-staining is recommended only for fixed cells. Appropriate fluorescent dyes or fluorescent protein expression systems for use in live-cell assays must be determined by the end user.

1. At the desired time-point after plating, remove culture media and fix cells with 3.7% formaldehyde in DPBS (or preferred fixative) using 250 µL/well. Incubate for 30 minutes at room temperature, protected from light.

2. Remove formaldehyde solution (dispose of as hazardous waste) and rinse twice with DPBS using 500 µL/well. Samples may be stored at this point in DPBS at 2-8°C, protected from light.

3. Prepare desired blocking/permeabilization buffer for fluorescent staining, e.g., DPBS with 2% blocking serum + 0.25% Triton X-100.

4. Thaw FITC-phalloidin to room temperature and reconstitute complete vial (20 µg) with 200 µL methanol to create 100 µg/mL stock solution. Remaining stock solution may be aliquotted and stored at -20°C, protected from light.

5. Immediately before staining, remove DPBS and rinse samples twice with fluorescent staining buffer using 500 µL/well.

6. Dilute FITC-phalloidin and DAPI in fluorescent staining buffer. Use FITC-phalloidin at a final concentration of 2 µg/mL (1:50) and DAPI at 1 µg/mL (1:100).
    a. Mix 128 µL of 100 µg/mL FITC-phalloidin + 64 µL of 100 µg/mL DAPI + 6208 µL fluorescent staining buffer = 6400 µL total staining solution.

7. Add 200 µL of staining solution to each well; incubate 1 hour at room temperature, protected from light.

    Note: If utilizing a primary/secondary antibody stain in addition to the phalloidin and DAPI, after Step 5, incubate with primary antibody alone in fluorescent staining buffer for 1 hour at room temperature, protected from light. Rinse samples three times with fluorescent staining buffer. Add secondary antibody to phalloidin/DAPI mixture and complete incubation as directed.

8. Remove staining solution (dispose of as hazardous waste); rinse twice with fluorescent staining buffer using 500 µL/well.

9. Rinse twice with DPBS using 500 µL/well.

10. For chamber slides, remove chambers and cover with slide mounting media (preferably containing anti-fade reagent) and appropriate-thickness cover glasses for magnification/imaging modality of choice. Allow mounted cover glasses to hard-set, or seal wet mounting media, as appropriate.
    a. For multi-well plates, leave in the last DPBS rinse for imaging.
    b. For coverslips, use slide mounting media to invert coverslips ("coated side" down) onto a glass slide or cover glass base for imaging. Allow mounted coverslips to hard-set, or seal wet mounting media, as appropriate.
    c. Stained samples may be stored at 2-8°C, protected from light.

Imaging and Analysis:

1. Perform fluorescent microscopic imaging of samples using appropriate fluorescence filters as listed in Table 2 below.

Fluorescent Label	Excitation Wavelength	Emission Wavelength
Cy3-Gelatin	550 nm	570 nm
FITC-Phalloidin	494 nm	518 nm
DAPI	358 nm	461 nm

Table 2. Fluorescence filter specifications.

2. Selection of imaging magnification and/or modality (e.g., widefield or confocal fluorescence) will depend upon application of interest. Lower magnifications (e.g., 20X objective) may be sufficient for degradation quantification of larger numbers of cells per field of view, while co-localization studies may require higher magnification/resolution visualization (e.g., 63X oil objective). Optimal imaging settings/techniques must be determined by the end user.

3. Image analysis procedures will depend upon the application of interest and analytical software to be used. Some potential quantification parameters for invadopodia degradation include:
   - Percent degradation area of total cell area
   - Degradation area (percent of field of view) normalized to number of cells
   - Number of cells associated with degradation (percent of total)
   - Number of cells with invadopodia* (percent of total)
   - Number of invadopodia* per cell

   (* Definitions of "active invadopodia" may vary, including F-actin or cortactin puncta co-localizing with degradation, actin puncta co-localizing with positive Arp2/3 expression, etc.)

   An example of image analysis utilizing free, downloadable ImageJ software distributed by the NIH (Rasband, 1997-2011) is given in **Figure 1**. DAPI signal may be thresholded for high intensities, then analyzed as "particles" to provide a nuclear (cell) count. Similarly, phalloidin signal may be thresholded for high intensities to allow measurement of cell area. Conversely, fluorescent gelatin signal may be thresholded for low intensities to enable degradation area quantification.

   Optimal image analysis settings/techniques and sampling strategies (e.g., number of replicates, number of fields of view, number of cells to be imaged) must be determined by the end user.

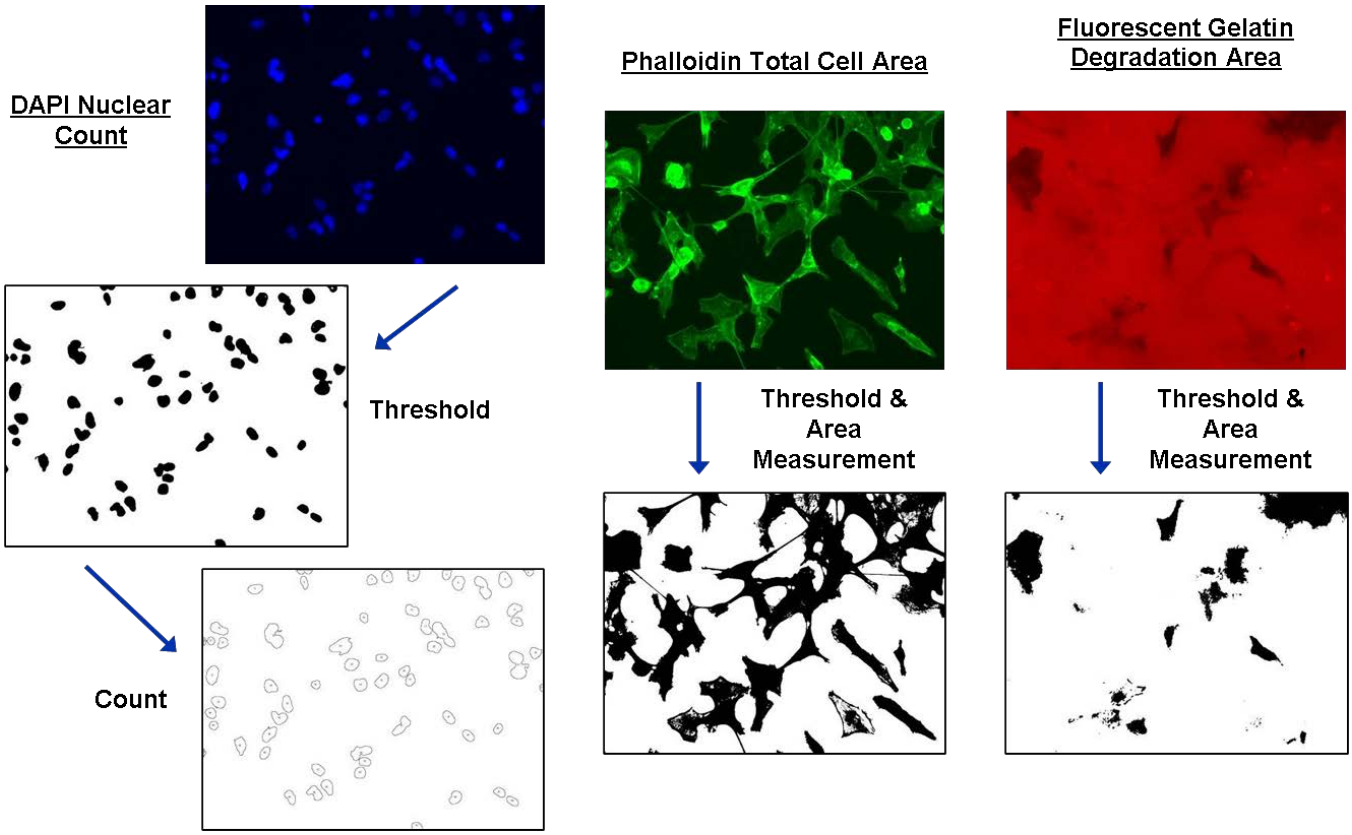


Figure 1. Example image analysis with NIH ImageJ software.

Example Data

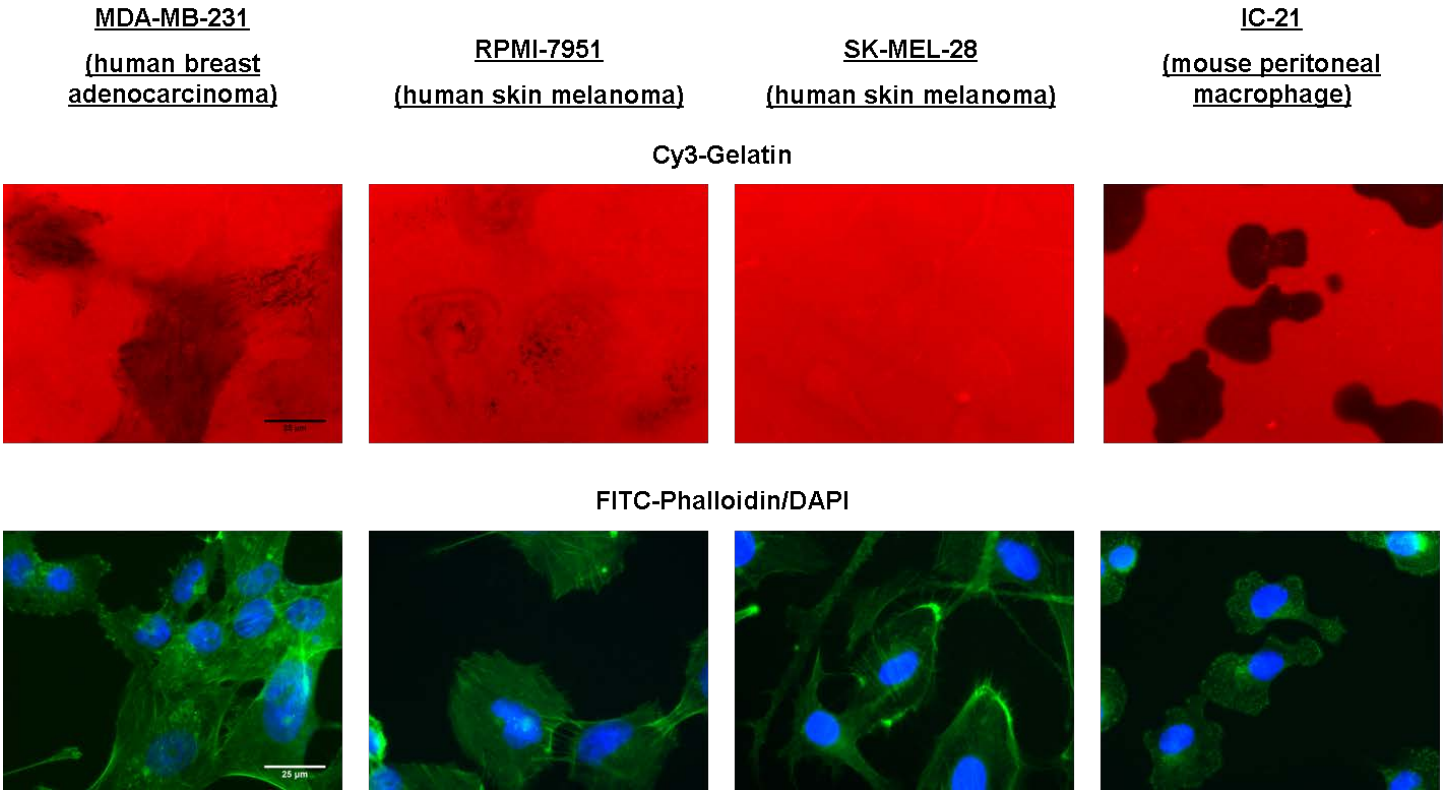
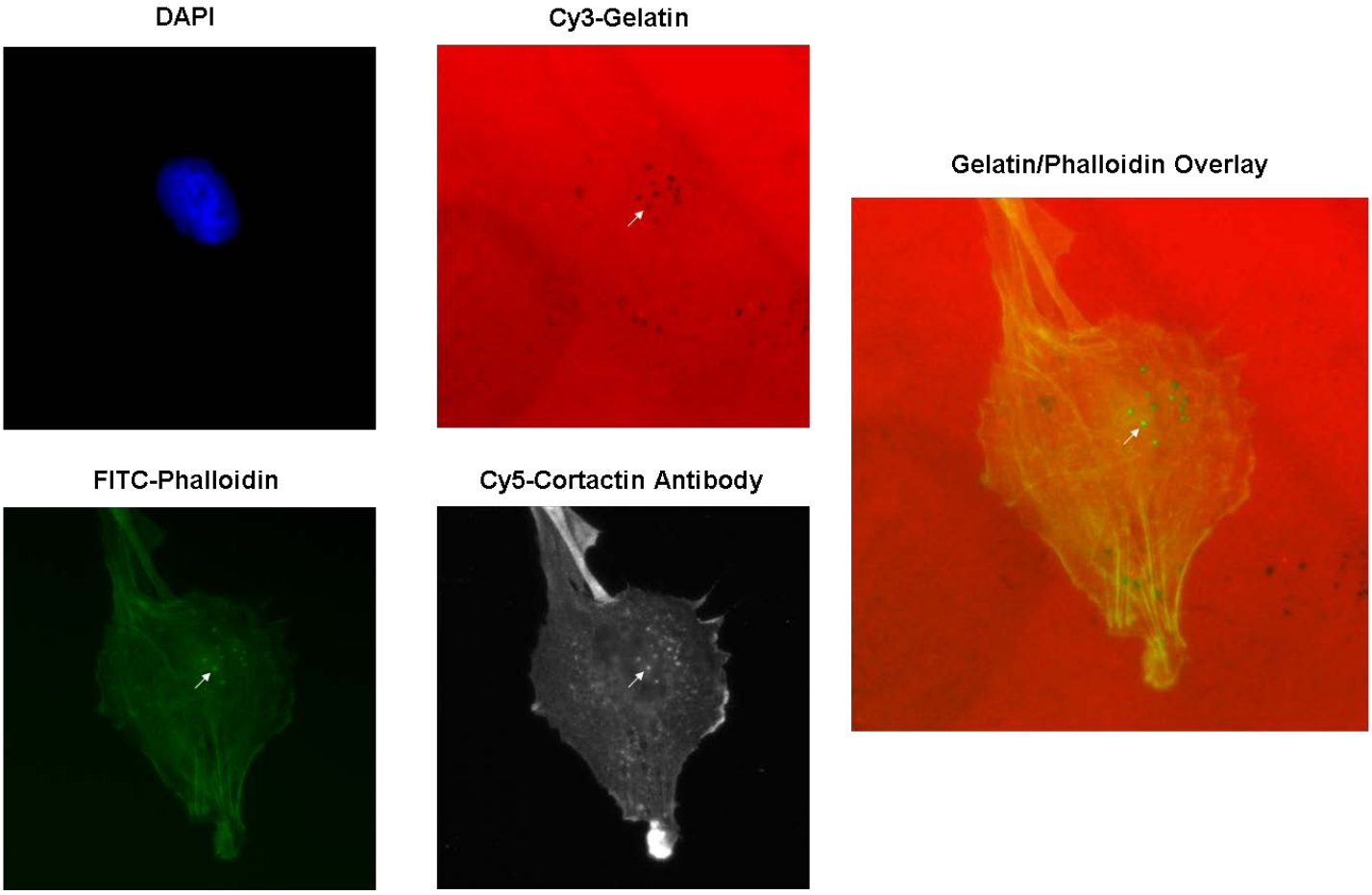


Figure 2. Fluorescent gelatin degradation and phalloidin/DAPI staining of multiple cell types.

Cy3-gelatin matrices were coated onto 8-well glass chamber slides as described in the **Assay Protocol**. Multiple human cancer cell lines (MDA-MB-231, RPMI-7951 and SK-MEL-28) and a mouse macrophage cell line (IC-21) were seeded onto the gelatin substrates at 20,000 cells/cm^2 for a culture duration of 24 hours. Fixation and staining with FITC-Phalloidin (*bottom panel*, green) and DAPI (*bottom panel*, blue) were performed according to the **Assay Protocol**. Cells were imaged on a widefield fluorescent microscope, with a 63X oil immersion objective lens. Bar = 25 µm.

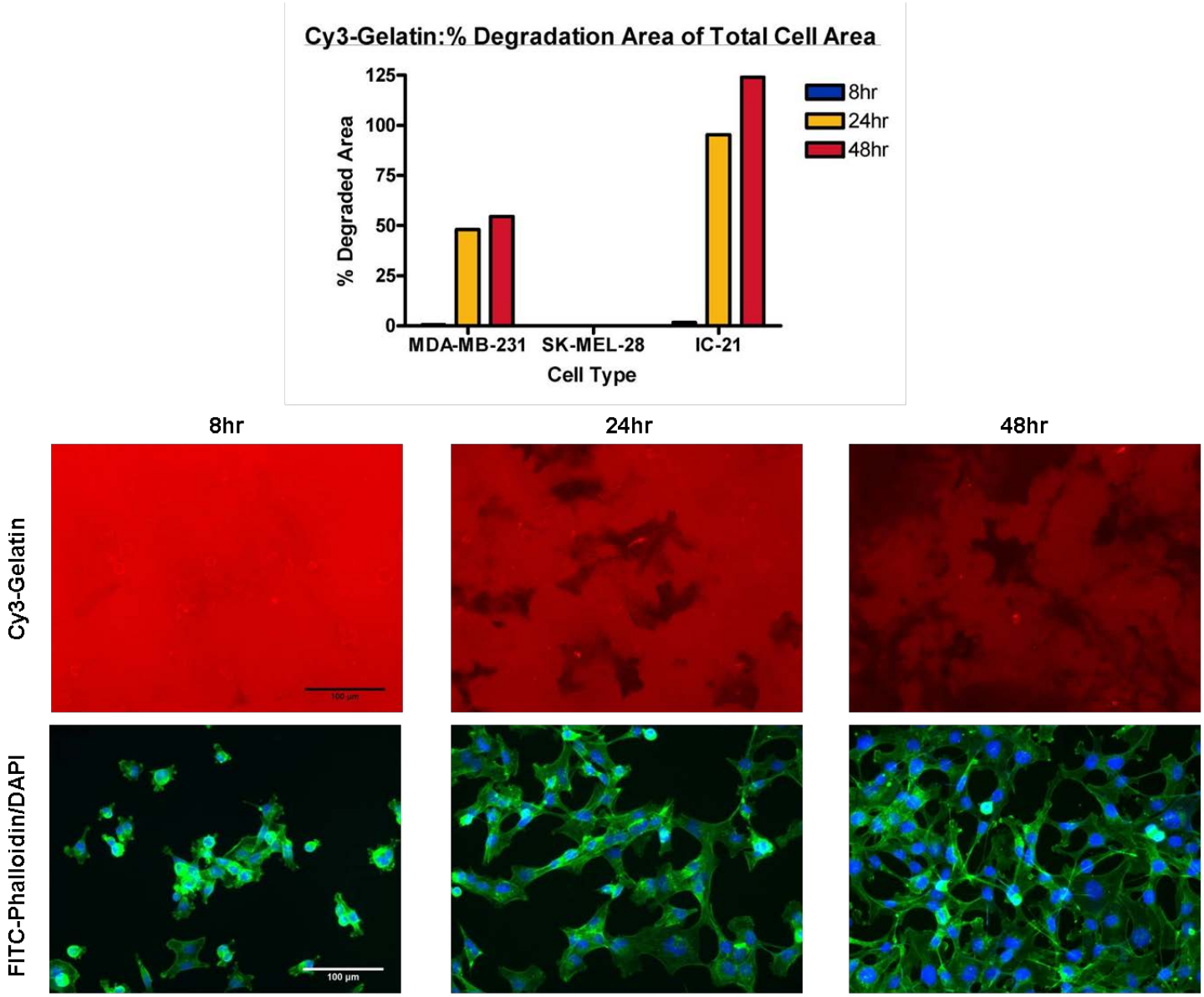
MDA-MB-231, RPMI-7951 and IC-21 gelatin degradation demonstrate the range of degradation patterns that may be observed due to invadopodia or podosome formation, including “punctate”, “linear” or “blotchy” areas devoid of Cy3 fluorescence. Often, not all cells in a population will exhibit proteolytic behavior, and cellular movement between sites of degradation may frequently be observed. SK-MEL-28 cells, a non-invasive melanoma type, do not display gelatin degradation.



<u>Figure 3.</u> Co-localization of degradation with invadopodia-related puncta.

Cy3-gelatin matrices were coated onto 8-well glass chamber slides as described in the **Assay Protocol**. RPMI-7951 human skin melanoma cells were seeded onto the gelatin substrates at 20,000 cells/cm^2 for 24 hours. Cells were fixed and incubated with a primary antibody against cortactin for 1 hour at room temperature. Following rinses with blocking/permeabilization fluorescent staining buffer, Cy5-secondary antibody incubation was performed concurrently with FITC-Phalloidin and DAPI staining, as detailed in the **Assay Protocol**. Cells were imaged on a widefield fluorescent microscope, with a 63X oil immersion objective lens.

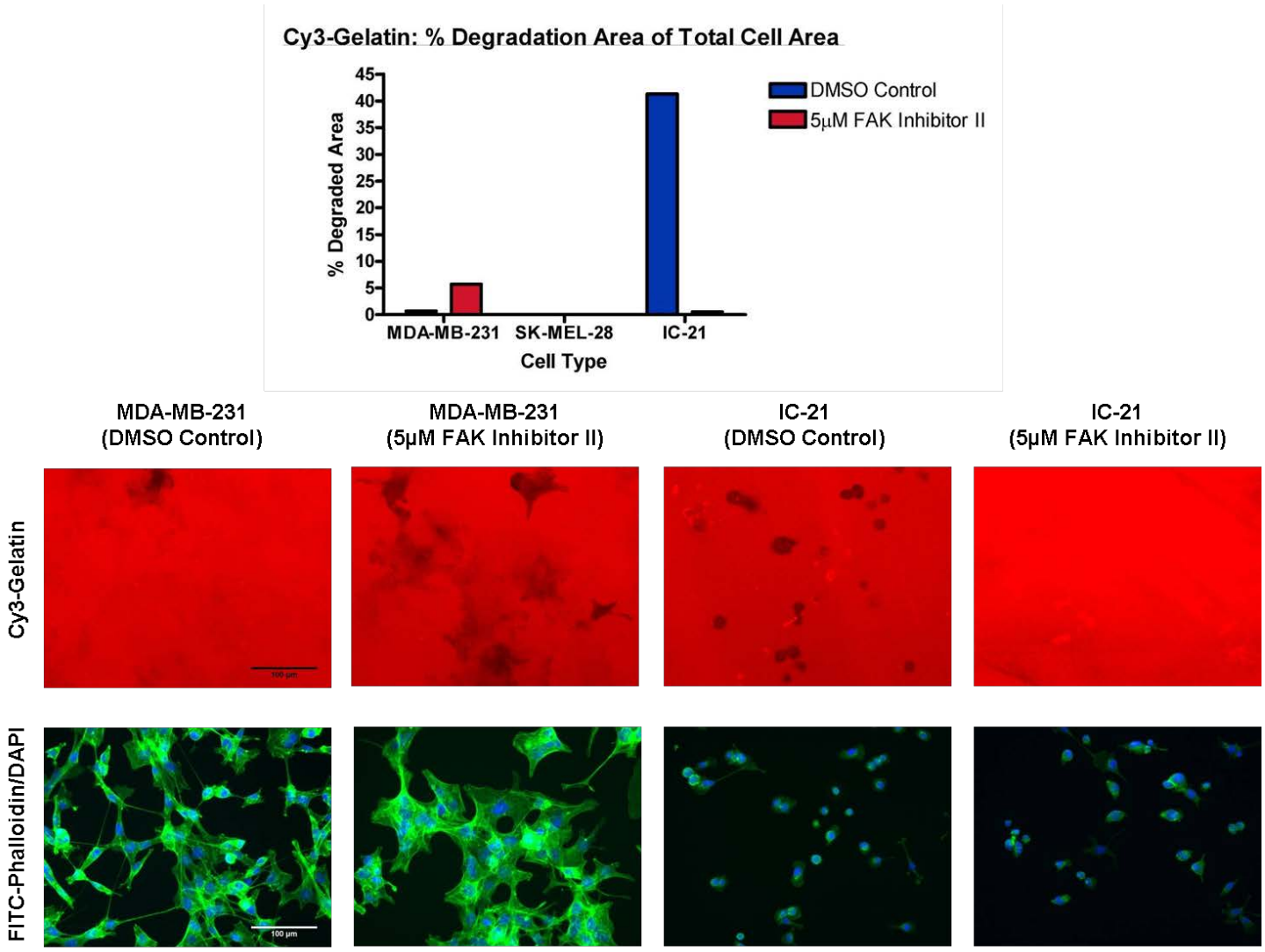
Indicative of “active” invadopodia, white arrows in the gelatin, phalloidin, cortactin and overlay images demonstrate an example of co-localization between matrix degradation, F-actin puncta and cortactin foci (cortactin protein is strongly associated with actin assembly and invadopodia formation).



<u>Figure 4.</u> Time-course of gelatin degradation.

Cy3-gelatin substrate preparation on 8-well glass chamber slides, cell fixation and FITC-Phalloidin (*bottom panel*, green) and DAPI (*bottom panel*, blue) staining were performed as described in the **Assay Protocol**. Parallel samples of MDA-MB-231, SK-MEL-28 or IC-21 cells seeded onto the gelatin substrates at 20,000 cells/cm^2 were cultured for durations of 8, 24 or 48 hours. Cells were imaged on a widefield fluorescent microscope, with a 20X air objective lens (5 fields of view per well). Bar = 100 µm.

Over 100 cells per condition were included in analysis of percent degradation area of total cell area. Image analysis was performed utilizing ImageJ software, as depicted in **Figure 1**. For MDA-MB-231 and IC-21 cells, degradation percentage increased over time, whereas no degradation by non-invasive SK-MEL-28 cells was observed at any time point. Note that although the theoretical maximum of percent degradation area of total cell area is 100%, higher amounts of degradation may be measured due to cellular movement during proteolysis (i.e., a migrating cell may produce a “path” of degradation of greater area than the cell itself).



<u>Figure 5.</u> Modulation of gelatin degradation by a focal adhesion kinase inhibitor.

Cy3-gelatin substrate preparation on 8-well glass chamber slides, cell fixation and FITC-Phalloidin (*bottom panel*, green) and DAPI (*bottom panel*, blue) staining were performed as described in the **Assay Protocol**. MDA-MB-231, SK-MEL-28 or IC-21 cells were seeded onto the gelatin substrates at 20,000 cells/cm^2 and simultaneously treated with 5 µM focal adhesion kinase (FAK) inhibitor II (PF-573,228) or a 0.4% DMSO control. Following 24 hour treatment, cells were fixed, stained and imaged on a widefield fluorescent microscope, with a 20X air objective lens (5 fields of view per well). Bar = 100 µm.

Over 100 cells per condition were included in analysis of percent degradation area of total cell area. Image analysis was performed utilizing ImageJ software, as depicted in **Figure 1**. FAK inhibition, which has previously been shown to increase invadopodia formation in certain cell types (Liu et al., 2010), was observed to increase MDA-MB-231 degradation under the tested treatment conditions. The non-invasive phenotype of SK-MEL-28 cells was not altered by addition of FAK inhibitor II, but IC-21 degradation was conversely decreased by treatment. Such opposite effects as those seen between the MDA-MB-231 and IC-21 cells emphasize variations in proteolytic behavior between cell types, and may be indicative of significant differences in degradation signaling mechanisms.

Troubleshooting

Problem	Possible Solutions
Undesirable coated gelatin matrix quality (signal intensity, homogeneity)	• Initial quality of glass surface may affect quality of coated matrix – consider glass cleaning step (e.g., acid wash) prior to poly-L-lysine coating, assuming wash compatibility will all materials associated with substrate (e.g., adhesive/gasket, well material for chamber slide); investigate differences in substrate manufacturing (sterilization method, pre-treatment for cell adhesion, etc.) to select substratum for best coating performance. • Glass chamber slides and dishes/multi-well plates may exhibit batch-to-batch variability in ability to support homogeneous matrix coating – try alternate batch of glass surface. • Adjust amount of fluorescently-labeled gelatin in mixture (i.e., ratio of labeled:unlabeled gelatin) to increase or decrease signal intensity from matrix, as appropriate for application of interest. • Warm fluorescent gelatin mixture to 60°C as described in Assay Protocol, Substrate Preparation and Cell Seeding, Step 7a to dissolve gelatin aggregates that may have accumulated during storage. • Optimize imaging conditions (magnification, numerical aperture, exposure time, gain, illumination intensity) for enhanced signal. • For highly surface-sensitive microscopy techniques (e.g., TIRF), thinner or dimmer gelatin layers may be desired – poly-L-lysine concentration or ratio of labeled:unlabeled gelatin may be modified (decreased) to optimize coating for these applications.
Insufficient or excessive gelatin matrix degradation	• Degree or presence of degradation can be highly cell type-dependent – test alternate cell types. • Cell lines can consist of subpopulations with different degrees of invadopodia formation, and the proportions of these subpopulations may change over time in continuous culture. Use each cell line at a defined passage number, and/or early passage cells from the same bank of cells, for best consistency between experiments. • Perform time-course of degradation for cell type of interest. • Degree or presence of degradation may be influenced by glass substrate properties – test alternate glass substrate formats or products.
No/minimal/unexpected effect by treatment with degradation modulator	• Modulator efficacy may vary with cell type – test alternate cell types. • Perform dose response or time-course of treatment on cell type of interest. • Consider specificity of treatment and potential off-target effects that may be influencing cell degradation behavior.

Problem	Possible Solutions
Poor staining quality (signal intensity, background)	• Increase concentration of blocking serum or protein in fluorescent staining buffer to decrease background. • Increase staining time or primary antibody/ secondary antibody/ phalloidin/ DAPI final staining concentrations to increase signal intensity. (Note: Higher staining intensity may be required for microscopic techniques involving high intensity illumination/rapid photobleaching, e.g., confocal or other laser illumination.) • Phalloidin and DAPI are largely cell impermeant – ensure presence and/or increase concentration of permeabilization agent (e.g., Triton X-100) in fluorescent staining buffer. • Protect fluorescent samples from light at all times and/or use anti-fade reagent (oxygen radical scavenger) in mounting media to minimize photobleaching. • Optimize imaging conditions (magnification, numerical aperture, exposure time, gain, illumination intensity) for enhanced signal.
Poor imaging focus	• Determine working (focal) distance of objective lenses used during microscopy and take into account when designing microscopy setup (i.e., imaging through glass slide/plate bottom vs. cover glass) – high magnification/oil immersion lenses may require thinner cover glasses (e.g., No. 0) for optimal focus. • Multiple imaging wavelengths (for Cy3, FITC, DAPI) may have different planes of focus depending upon extent of chromatic aberration correction in imaging lenses – consider imaging at multiple focal planes for optimal focus at each wavelength.
Inaccurate image analysis/ high error in analyzed data	• Refine image analysis strategy to improve ability of algorithm to eliminate analysis errors (e.g., enhance clump-breaking, debris exclusion, accuracy of thresholding limits, elimination of visual artifacts from analysis, etc.). • Optimize sampling strategy (e.g., increase number of replicates, fields of view, or cells imaged) to reduce noise/error in data.

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