Application Note

Fluorescent Gelatin Degradation Assays for Investigating Invadopodia Formation

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Abstract

The invasion of cells through tissue and associated extracellular matrix is a critical activity in both physiological and pathological processes, such as embryological development and cancer metastasis. A key cellular feature involved in matrix degradation is the formation of protrusions of localized protease activity, termed invadopodia or podosomes. An effective method for visualizing subcellular invadopodia formation involves the plating of cells onto a thin layer of fluorescently-labeled matrix. Areas of degradation are associated with a loss of fluorescence, and these regions may be microscopically imaged and colocalized with molecules of interest in the proteolytic pathway. Here we demonstrate the capabilities of two new invadopodia assay kits, which provide the reagents necessary for generating thin coatings of prelabeled fluorescent gelatin on glass substrates. These kits enable simple, rapid, and consistent production of homogeneous gelatin matrices and visualization of degradation produced by multiple cell types. Furthermore, degradation may be quantified by image analysis and used to characterize proteolytic time-courses and modulator effects on invadopodia formation.

Introduction

Invasion of cells through layers of extracellular matrix is a key step in tumor metastasis, inflammation, and development. Stages of invasion include 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[1].

Each of these stages 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 requires techniques with higher resolution.

The method that has been most informative for pinpointing regions of the cell that initiate invasion involves 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[2]. 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[3]. Many molecules orchestrate the formation and function of invadopodia; a few of the key molecular events include Src phosphorylation of scaffolding protein Tks5[4], N-WASP activation and cortactin regulation of the Arp2/3 complex to induce actin polymerization[5,6], generation of reactive oxygen species by NADPH oxidases[7], and cortactin-mediated localization of membrane-type and secreted matrix metalloproteases (MMPs) to the invadopodia[8].

Merck Millipore's QCM™ Gelatin Invadopodia Assays provide optimized materials and protocols to enable reproducible analysis of invadopodia in invasive tumor cells (Catalog No. ECM670 for green fluorescence, Catalogue No. ECM671 for red fluorescence). Reagents are provided for coating glass culture surfaces with fluorescent matrix and for colocalizing the actin cytoskeleton and nuclei with invadopodial degradation sites. This assay may also be used for assessing the activity of inhibitors and promoters of invadopodia formation and function. Furthermore, different cell types and individual cells in heterogeneous populations may be analyzed for invasive potential. Finally, the assay kits provide troubleshooting suggestions, recommendations for coating on multiple substrate formats, and example studies in several assay systems (e.g., various cell types, time-course studies, degradation modulation).

Assay Principle

The Merck Millipore QCM Gelatin Invadopodia Assays provide the reagents necessary for affixing a thin, uniform layer of pre-labeled fluorescein (green)- or Cy3 (red)-gelatin to a glass culture substrate, allowing for rapid detection of matrix degradation[9,10]. A poly-L-lysine coating is first adsorbed to the glass substratum. The substrate is then treated with a dilute glutaraldehyde solution to bifunctionally "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, and treatment compounds of interest may also be introduced within the culture period (Figure 1).

Degraded areas of gelatin, now devoid of fluorescence, may be microscopically visualized and quantified using image analysis software. The assay also provides fluorescently-labeled phalloidin (TRITC- or FITC-conjugated) and DAPI, for visualization of cytoskeletal F-actin and nuclei, respectively, to allow colocalization 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 mechanistic studies.

Materials and Methods

Cell Lines Used

MDA-MB-231 human breast adenocarcinoma, RPMI-7951 and SK-MEL-28 human skin melanoma, and IC-21 mouse peritoneal macrophages were obtained from ATCC® and cultured to 80-90% confluence in tissue culture flasks. For seeding onto gelatin surfaces, cells were detached using 0.25% trypsin-EDTA (or DPBS without calcium and magnesium for IC-21 cells), pelleted, then resuspended in growth medium to a concentration of 28,000 cells/mL (20,000 cells/cm^2). Cells were seeded in a volume of 500 µL/well and cultured for the desired duration of degradation, generally between 8-48 hours.

Substrate Preparation and Cell Seeding

To facilitate attachment of fluorescent gelatin, 8-well glass chamber slides were first coated with 250 µL/well of dilute poly-L-lysine in deionized water for 20 minutes at room temperature (RT). The poly-L-lysine was then removed, and the slides rinsed three times with 500 µL/well of Dulbecco's PBS (DPBS). Next, 250 µL of dilute glutaraldehyde in DPBS was added to each well for 15 minutes at RT to activate the poly-L-lysine surface for further protein attachment. Following removal of the glutaraldehyde, each well was again rinsed three times with 500 µL of DPBS. Finally, 200 µL of gelatin in DPBS, mixed at a 1:5 ratio of fluorescently-labeled:unlabeled gelatin, was coated onto each well for 10 minutes at RT, followed by three rinses in DPBS. All steps including, and subsequent to, fluorescent-gelatin coating were performed so as to protect the glass slides from photobleaching due to excessive exposure to light.

To prepare for cell plating, the gelatin substrates were disinfected with 500 µL/well of 70% ethanol for 30 minutes at RT. After ethanol removal and rinsing in DPBS, free aldehydes were quenched by the addition of 500 µL/well of amino-acid-containing growth medium and incubated at RT for 30 minutes. Cell types of interest were detached and seeded as described in the previous section. For some experiments, a modulator of invadopodia formation, focal adhesion kinase inhibitor II (5 µM final concentration, or 0.4% DMSO control), was added simultaneously with plating.

Sample Fixation and Staining

At the desired time-point after plating, growth medium was removed from the chamber slides and the samples were fixed for 30 minutes at RT with 250 µL/well of 3.7% formaldehyde in DPBS. Samples were then rinsed twice with 500 µL/well of fluorescent staining buffer (DPBS with 2% blocking serum and 0.25% Triton X-100 for cell

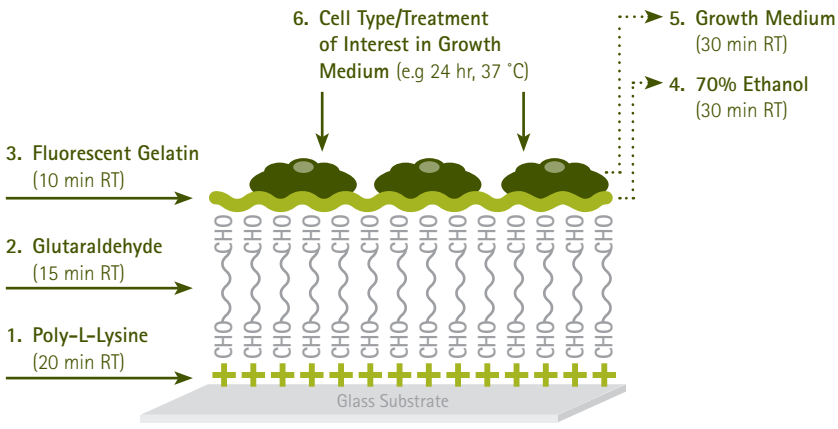


Figure 1. Gelatin invadopodia assay setup.

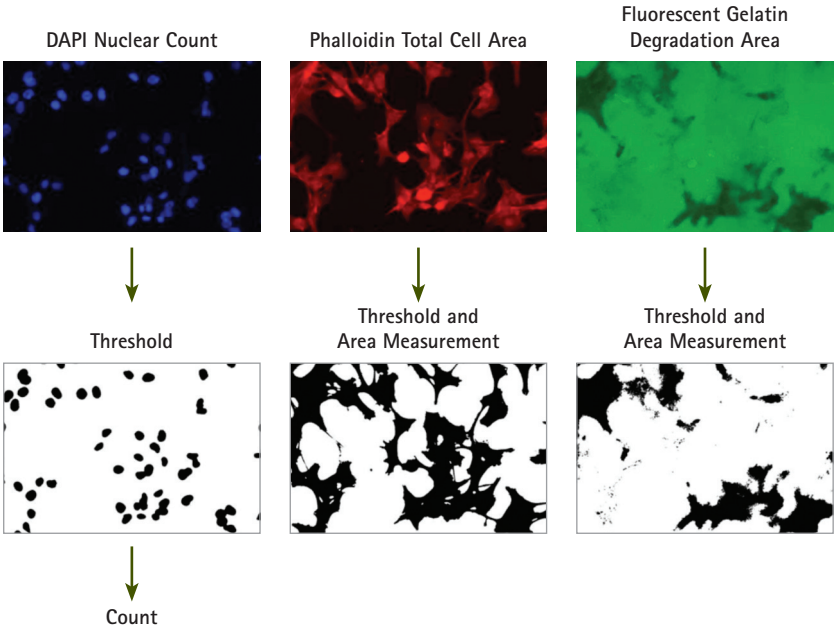


Figure 2. Example image analysis with NIH ImageJ software.

permeabilization). For immunocolocalization studies, 200 µL of primary antibody in fluorescent staining buffer was added to each well for 1 hour incubation at RT. Samples were then rinsed three times with 500 µL/well of fluorescent staining buffer before proceeding on to 1 hour RT incubation with fluorescent secondary antibody, fluorescently-conjugated phalloidin (2 µg/mL) and DAPI (1 µg/mL) in staining buffer. Primary and secondary antibodies were omitted for stains incorporating phalloidin and DAPI only. Finally, samples were rinsed twice each with fluorescent staining buffer and DPBS before removal of culture chambers and cover-slipping. Slide-mounting medium contained anti-fade reagent and appropriately thick cover glasses were selected for imaging magnification of choice.

Imaging and Analysis

Mounted cover glasses were allowed to hard-set before fluorescent imaging with illumination and filters appropriate for fluorescein/FITC, Cy3/TRITC and DAPI excitation and emission wavelengths. Samples were imaged on an inverted wide-field fluorescent microscope at 20X objective magnification for quantification studies (5 fields of view per well) or at 63X objective magnification (oil immersion) for colocalization experiments.

Image analysis was performed using free, downloadable ImageJ software distributed by the National Institutes of Health (NIH)[10]. A high intensity threshold was set for positive DAPI signal, then analyzed as "particles" for determination of a nuclear (cell) count. Similarly, setting a high intensity threshold for phalloidin signal enabled measurement of total cell area per field of view. Conversely, a low intensity threshold was set for diminished fluorescent gelatin signal to enable quantification of total degradation area per field of view (Figure 2).

Results and Discussion

We demonstrated the ability to visualize and quantify the degradation of fluorescent gelatin matrices by a variety of cell types. For the cell lines MDA-MB-231, RPMI-7951, and IC-21, gelatin proteolysis demonstrated a range of degradation patterns that may be attributed to invadopodia or podosome formation, including "punctate", "linear", or "blotchy" areas devoid of fluorescein-gelatin fluorescence (Figure 3). 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 noninvasive melanoma type, did not display gelatin degradation (Figure 3).

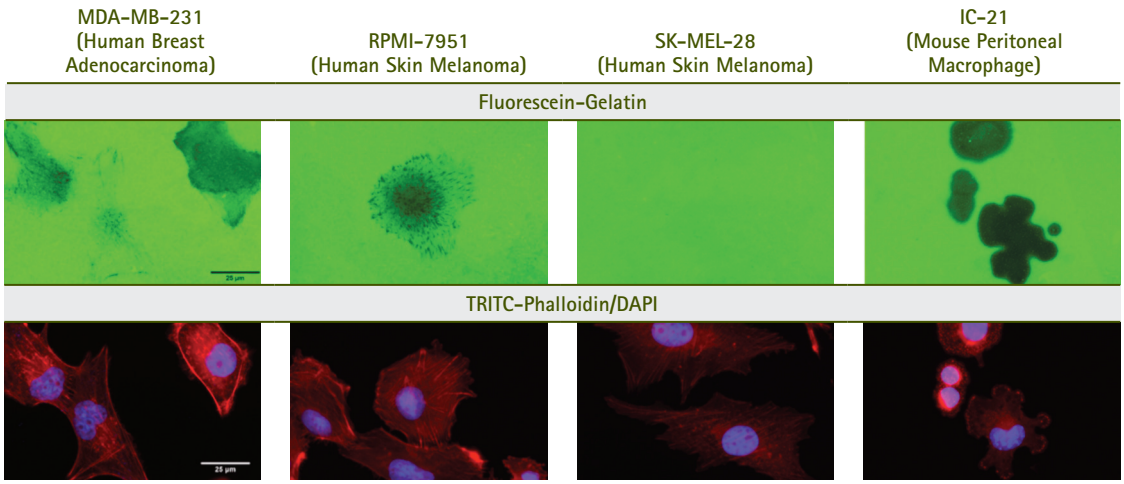


Figure 3. Fluorescent gelatin degradation and phalloidin/DAPI staining of multiple cell types. Fluorescein-gelatin matrices (top panel, green) were coated onto 8-well glass chamber slides. Multiple human cancer cell lines (MDA-MB-231, RPMI-7951, and SK-MEL-28) and a mouse macrophage cell line (IC-21) were plated onto the gelatin substrates at 20,000 cells/cm^2 for a culture duration of 24 hours. F-actin and nuclei were stained, respectively, with TRITC-phalloidin (bottom panel, red) and DAPI (bottom panel, blue). Cells were imaged at 63X objective magnification (bar = 25 µm).

Colocalization Studies

In Figure 4, RPMI-7951 cells seeded onto Cy3-gelatin matrices demonstrated the ability to co-localize sites of gelatin degradation with phalloidin (F-actin) puncta and cortactin foci. Cortactin protein is strongly associated with actin assembly, and colocalization of this molecule with areas of proteolysis was indicative of dynamically "active" invadopodia formation (see white arrows in figure 4).

Time-dependent Degradation

Over 100 cells per condition were analyzed to obtain the "percent degradation area of total cell area" data depicted in Figure 5. For MDA-MB-231 and IC-21 cells, degradation percentage increased over time (particularly between 8 and 24 hour time points), whereas no degradation by noninvasive SK-MEL-28 cells was observed. Of note is that although the theoretical maximum of "percent degradation area of total cell area" is 100%, higher amounts of degradation were observed here, likely due to cellular movement during proteolysis. Such "historical" degradation is recorded using this assay, resulting in degradation areas larger than the area of a cell itself (particularly for longer time points or highly motile cell types).

Modulation of Matrix Degradation

Cells were seeded onto fluorescein-gelatin matrices and simultaneously treated with focal adhesion kinase (FAK) inhibitor II (PF-573,228) or a DMSO control (Figure 6). FAK inhibition, which has previously been shown to enhance invadopodia formation in certain cell types[12], was indeed observed to increase MDA-MB-231-associated degradation over the course of 24-hour treatment. The non-invasive phenotype of SK-MEL-28 cells was not altered by addition of FAK inhibitor II, but surprisingly, degradation by IC-21 cells was decreased by treatment with the compound. 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 between cancerous and normal cell phenotypes.

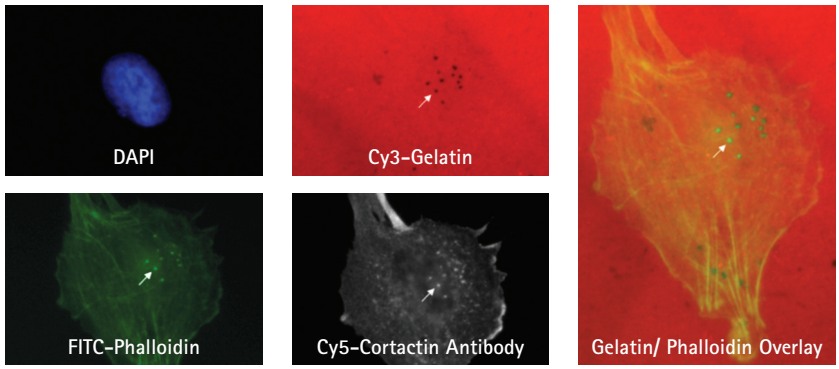


Figure 4. Colocalization of degradation with invadopodia-related puncta. Cy3-gelatin matrices were coated onto glass chamber slides, and RPMI-7951 human skin melanoma cells were seeded onto the gelatin substrates for 24 hours. For fluorescent immunocytochemistry, cells were incubated with anti-cortactin, followed by detection with a Cy5-conjugated secondary antibody. Secondary antibody incubation was performed concurrently with FITC-phalloidin and DAPI staining. Cells were imaged at 63X objective magnification.

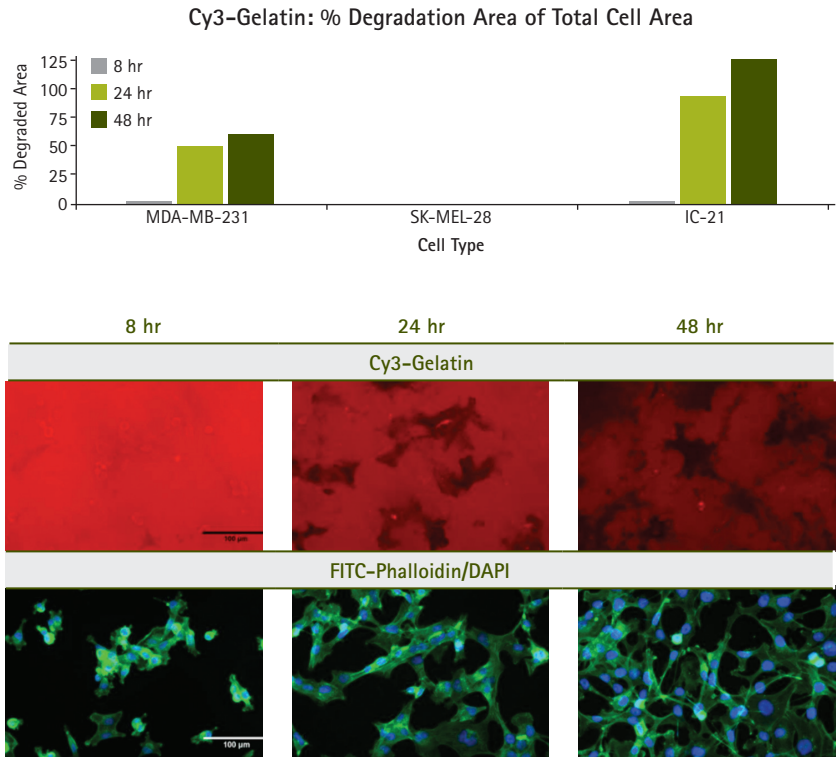
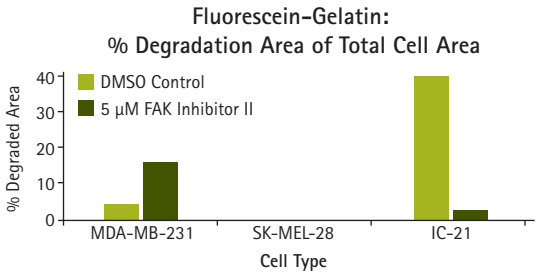


Figure 5. Time-dependent gelatin degradation. Multiple cell types (images are of MDA-MB-231) were plated onto Cy3-gelatin substrates (top image panel, red) and cultured for 8, 24, or 48 hours. Following staining with FITC-phalloidin (bottom image panel, green) and DAPI (bottom image panel, blue) cells were imaged at 20X objective magnification at 5 fields of view per well. Bar = 100 µm. "Percent degradation area of total cell area" was quantified using ImageJ analysis software, as depicted in Figure 2.

Conclusion

Our results demonstrate the utility of Merck Millipore's QCM Gelatin Invadopodia Assays in the visualization and quantification of gelatin degradation by a variety of cell types at multiple time points and following treatment with modulators of invadopodia formation. Specifically, we show that both time-dependence of matrix degradation and efficacy of invadopodia modulators are heavily influenced by cell type, inviting further, detailed studies of the differential signaling controlling these processes. To this end, QCM Gelatin Invadopodia Assays can provide a convenient, flexible system for monitoring matrix degradation and investigating key players in the proteolytic process, at the single cell and subcellular levels.



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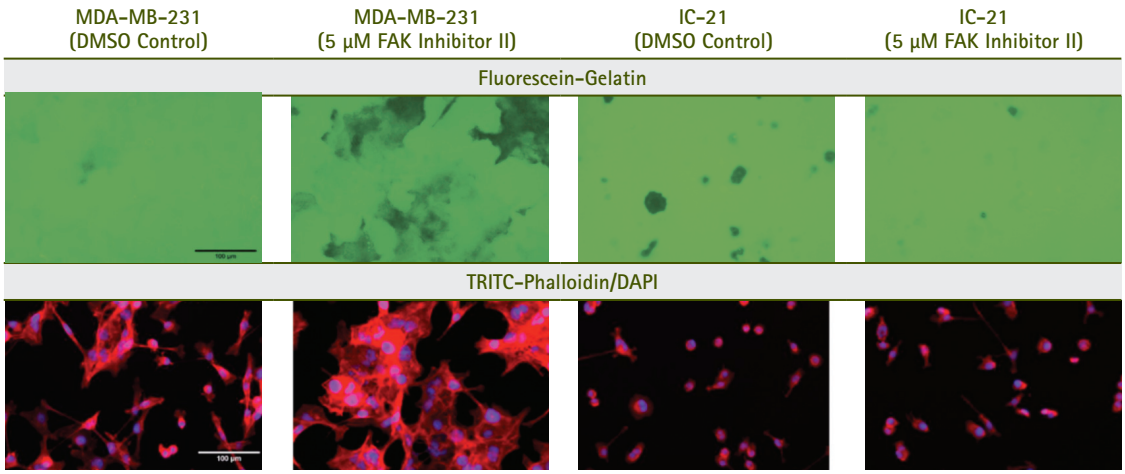

Figure 6. Modulation of gelatin degradation by a FAK inhibitor. Fluorescein-gelatin matrices (top image panel, green) were seeded with MDA-MB-231, SK-MEL-28, or IC-21 cells and simultaneously treated with 5 µM FAK inhibitor II or a 0.4% DMSO control. Following 24 hour treatment, cells were fixed and stained for F-actin and nuclei with TRITC-phalloidin (bottom image panel, red) and DAPI (bottom image panel, blue). Samples were imaged at 20X objective magnification at 5 fields of view per well. Bar = 100 µm.

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