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Visualizing Protease Activity in Living Cells: From Two Dimensions to Four Dimensions

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Abstract

Proteolytic degradation of extracellular matrix (ECM) components by cells is an important metabolic activity as cells grow, remodel, and migrate through the ECM. The ability to analyze ECM degradation can be valuable in the study of developmental processes as well as pathologies such as cancer. In this protocol we describe an in vitro live cell-based method to image and quantitatively measure the degradation of ECM components by live cells. Cells are grown in the presence of fluorescent dye-quenched protein substrates (DQ-gelatin, DQ-collagen I, and DQ-collagen IV) that are mixed with protein matrices. Upon proteolytic cleavage, fluorescence is released that directly reflects the level of proteolysis by the cells. Using confocal microscopy and advanced imaging software, the fluorescence is detected and accurate measurements of proteolytic degradation in three and four dimensions can be assessed.

Keywords

3-D culture; recombinant basement membrane; DQ-substrates; proteases; live cell imaging; ECM

INTRODUCTION

In vivo, cellular microenvironments are constructed of complex combinations of extracellular matrix (ECM) proteins that are under constant turnover and modification. During most forms of cellular motility, cells remodel their immediate microenvironment via proteolytic degradation of ECM (Friedl and Wolf, 2003). Thus, the ability to examine the proteolytic degradation of ECM by living cells is crucial for understanding many aspects of cellular motility in normal processes and in pathologies such as cancer. The assay described here allows one to quantitatively analyze extracellular and intracellular proteolysis associated with degradation of ECM surrounding the cells. This is a microscopy-based assay in which proteolysis of fluorogenic dye-quenched protein substrates (DQ-substrates) by live cells is imaged. The cells are grown as either two dimensional (2-D) or three-dimensional (3-D) cultures on ECM or embedded in ECM, respectively. The DQ-substrates are mixed into the ECM and provide a protein substrate that can be cleaved by many proteases. This differs from the use of small molecule synthetic substrates that are cleaved selectively by a single protease or protease class.

Originally, the DQ-substrates were developed as non-selective, broad-spectrum substrates for in vitro applications such as analysis of protease activity in solution (Menges et al., 1997). We have adapted their use for live-cell confocal imaging in order to investigate the contribution of multiple classes of proteases to ECM degradation by migrating or invading cells. Visualization and accurate quantitative analysis of ECM proteolysis relies on the fluorescent characteristics of the DQ-substrates. The native, undegraded DQ-substrates are

heavily conjugated with the fluorogenic dye, fluorescein. The close proximity of the dye molecules to each other renders native DQ-substrates fluorescently quenched due to a Fluorescence Resonance Energy Transfer (also known as Forster Resonance Energy Transfer or FRET) effect. Upon proteolytic hydrolysis of the DQ-substrate into smaller fragments, the fluorogenic dye molecules are separated, self-quenching is lost, and a strong fluorescence is emitted. The fluorescent signal is proportional to the proteolytic activity, and the pattern depends on the DQ-substrate itself. There is little background fluorescence in the absence of cells for either DQ-collagen I (Fig. 4.20.1A) or DQ-collagen IV (Fig. 4.20.1C). The cellular cleavage of DQ-collagen I (Fig. 4.20.1B) or DQ-collagen IV (Fig. 4.20.1D) differs with the former appearing as fluorescent dots along the collagen I fibrils and the latter as diffuse fluorescence. Depending on the cell lines and ECM, cells will grow as either 2-D monolayers, e.g., on gelatin and collagen I, or as 3-D structures on complex matrices such as reconstituted basement membrane (rBM) (Figure 4.20.2). The ECM can be mixed with any one of an assortment of DQ-substrates (DQ-albumin, DQ-gelatin, DQ-collagen I, or DQ-collagen IV) available through Invitrogen, thus creating customized combinations through which to analyze proteolysis in the context of a defined ECM. Recent studies from our laboratory and others employing this assay have shown that normal and tumor cells use several classes of proteases to actively degrade collagen IV and collagen I and, depending on the cell type, the degradation products are found intracellularly and/or extracellularly (Sameni et al., 2003; Cavallo-Medved et al. 2005; Podgorski et al. 2005; Tsai et al. 2005; Urbich et al. 2005; Sloane et al., 2006). We describe here the use of three combinations of matrices: (1) DQ-collagen I in collagen I; (2) DQ-gelatin in gelatin; and (3) DQ-collagen IV in recombinant basement membrane (rBM; Support Protocol 1).

BASIC PROTOCOL 1

QUANTITATIVE MEASUREMENT OF PROTEOLYTIC DEGRADATION OF DQ-SUBSTRATES IN LIVE-CELL CULTURES USING A LASER SCANNING CONFOCAL MICROSCOPE

Cells are grown on ECM containing DQ-substrates as described in Support Protocol 1. Although either an inverted or an upright laser scanning confocal microscope can be used for imaging live cells, this assay is most readily performed using an upright microscope. An upright microscope is ideally suited due to the inherent thickness (1–3 mm) of the culture sample (DQ-substrate in ECM + cells). Regardless of the microscope, the sample thickness requires the use of objectives with long working distances. For live-cell imaging at high magnification on an upright microscope, we routinely use water-immersion dipping objectives (40× and 63× Achromat) that can be immersed in the cell culture medium. Note that these are different than the normal high-power Apochromat water-immersion objectives. The latter have very-short working distances (10–15 μm) and cannot be used with thick specimens to obtain a sharp focus through the entire sample. For imaging with an inverted microscope, one will use either a lower magnification lens or a long-working-distance lens (see Commentary). In our laboratory, we routinely use a Zeiss upright microscope equipped with Zeiss LSM 510 META confocal optics and argon, HeNe and two-photon lasers. Together with a microscope stage incubator, this setup allows a high level of flexibility in regard to sample dimensions, imaging timeframes, and fluorogenic dyes.

The key to quantitative imaging of proteolysis in three dimensions is the ability to acquire fluorescence without pixel saturation from single planes of focus encompassing the entire specimen. Confocal microscopy allows us to image sequentially above, through, and below the objects of interest. This optical *z*-sectioning and imaging of accumulated fluorescence gives a spatial 3-D output that represents proteolysis of DQ-substrates. Furthermore, the same field of view can be imaged repeatedly over a period of time to collect data in four dimensions (4-D), i.e., spatially and temporally. Regardless of whether data collection is

over time or just a snapshot of one time point, accurate determination of proteolytic activity and localization of such activity requires the collection of data from all the cells and their surrounding 3-D volume (x,y,z -limits). As some cell types aggressively invade into the ECM, the optical sections may need to span several hundred micrometers in the z -direction. Therefore, prelabeling the cells (Support Protocol 1) and their nuclei (Basic Protocol 1) with fluorescent dyes will enable the user to not only define the entire relevant 3-D volume, but also to relate total DQ-substrate hydrolysis to cell number. In addition, prelabeling the cells with cell tracking dyes that occupy the entire cell enables differentiation of intracellular from extracellular proteolysis and their quantification (Basic Protocol 2). The additional data from nuclear and cytoplasmic staining allows normalization of proteolysis to cell number across samples. Visually, most advanced imaging applications are capable of transforming the collection of 2-D images from optical z -sections to graphical 3-D reconstructions of cells and areas of DQ-substrate degradation (Figure 4.20.3).

Materials

Prelabeled cell cultures established in ECM containing DQ-substrates (Support Protocol 1), grown on coverslips

Upright laser scanning confocal microscope equipped with a water-immersion lens and appropriate filter sets and lasers

Microscope stage incubator with humidity, temperature, and CO₂ control

Cell-permeable DNA-binding dye [Hoechst 33342 (Invitrogen) or DRAQ5 (Biostatus Ltd)]

Cell culture medium

35-mm cell culture dish

Prepare for imaging

- 1 Set up the microscope and stage incubator.

In order to avoid power fluctuations during early imaging, turn on the microscope lasers for at least 5 min prior to imaging. When imaging over extended periods of time it is important to equilibrate the stage incubator to 37°C and 5% CO₂ and 40% to 50% non-condensing humidity.

- 2 Add DNA-binding fluorescent dye (Hoechst 33342) to a final concentration of 5 µg/ml in culture medium.

When performing live-cell imaging over extended periods of time, the time to add the DNA dye is an important consideration. Some DNA binding agents like Hoechst 33342 and Draq5 are not toxic over short periods of time, yet may have adverse effects on the cells over longer times (hours). Therefore, it is recommended that these dyes be added to the culture medium 10 minutes before short-term imaging (<1 hr) or 1 hr before the end of long term imaging. Alternatively, cells can be engineered to express a nuclear- targeted fluorescent protein.

- 3 Place the 35-mm cell culture dish on the microscope stage.

Ensure that the coverslip is located in the center of the culture dish. This will allow ample room for the objective to navigate the entire surface of the coverslip.

- 4 Identify the structures of interest.

Choose structures that are representative of the population being analyzed. The thickness of structures being imaged should not exceed the focal limit of the microscope.

Establish imaging parameters

- 5 Using a fast-scanning mode on the green channel (excitation 488 nm, emission 521 nm), bring structures into the focal plane with the brightest fluorescence.
- 6 Scan an image of the structure at the desired excitation light intensity, line averaging, zoom, etc.
- 7 Adjust the detector pinhole diameter to one airy unit.
- 8 Modify detector gain for maximal fluorescence signal without pixel saturation (pixel intensities that exceed the detector scale, i.e., >255 for an 8-bit image).

Saturated pixels only register as the maximum detector value, 255, so the true pixel intensity cannot be calculated under these conditions. Detector gain and offset will vary depending on the DQ-substrate used (type/lot number) and the laser power. The imaging parameters should be held constant for all z-sections through the 3-D structures.

- 9 Repeat the same adjustments (steps 5 through 8) for the other channels used.
- 10 Define the z distance limits, including the first and last optical sections of a stack as well as the z-stack interval distance between optical sections.

Use a fast-scan mode along with the manual focus adjustment to determine the focal plane in which the fluorescence signal from the DQ-substrate begins and ends. This is usually near the top and bottom of the cellular structures. These positions will be different for each structure imaged and will have to be determined for each structure. Larger structures and highly motile cells will require a greater number of sections to image the entire volume (x,y,z). Although the number of sections will change from structure to structure, the thickness of each section and distance between z-sections must be kept consistent in order to accurately compare structures.

Collect fluorescence data

- 11 Create a z-stack of images including scans for the DQ-substrate, DNA binding dye, and cell tracking dye.

At each focal plane the fluorescence corresponding to all channels is collected. The green fluorescence represents cleaved DQ-substrate. The fluorescence from cytoplasmic cell tracking dyes such as CellTracker™ will be used to identify cellular boundaries. The fluorescence from DNA-binding dyes will define the nuclear area in all cells not in late mitosis and allow determination of cell number.

- 12 Collect at least two to four different z-stacks of representative cellular structures and surrounding matrix from each experimental condition and treatment.

BASIC PROTOCOL 2

QUANTITATIVE ANALYSIS OF CONFOCAL IMAGE Z-STACKS

Following the acquisition of data in the form of z-stacks, image analysis software is used for quantification. The fluorescence data acquired due to the proteolytic cleavage of DQ-

substrates can be used to visualize DQ-substrate proteolysis and quantify proteolysis in particular areas or entire volumes (Fig. 4.20.4).

Materials

Computer system capable of processing large image files: 350 MHz or faster processor, large storage capacity, and at least 256 MB video RAM and minimum 1 GB system RAM

Advanced image processing/analysis software capable of applying thresholds and measuring pixel intensities [e.g., ImageJ (Open Source, Public Domain), MetaMorph (Molecular Devices) and Volocity (Improvision)].

Process the confocal image stacks for analysis

1 Export confocal z-stacks

This is performed only if the microscope software saves image stacks in a format that cannot be read by the available image analysis software. It is important to export in a format that retains all image information. We recommend that images be exported as uncompressed Tagged Image File Format (TIFF). This file format will be accepted by all image analysis software.

2 Open an image stack in its original file format or reassemble the exported TIFF files as an image stack.

Almost all advanced image processing software packages can assemble a series of 2-dimensional images into a 3-dimensional image stack, provided the image names end in sequential numbers designating their positions in the stack.

3 Separate the DQ-substrate channel, the cell tracker channel, and the DNA-binding dye channel.

The 2-dimensional images containing data from DQ-substrate cleavage, cell tracking, and DNA-binding dyes are all captured on different channels. Fluorescence resulting from the cleavage of DQ-substrates is captured on the green channel due to the emission spectrum of the fluorochrome (FITC). In order to quantify the image information from this channel, it must first be separated from all other channels and processed for analysis. The result of separation will be three 8-bit grayscale image stacks. The green channel stack will yield the total (extracellular and intracellular) degradation information. The red channel will be used to delineate the intracellular cytoplasmic area of cells and be used to separate intracellular from extracellular areas in the green channel (step 7). The blue channel will be used to determine the number of cells as long as each cell contains only one nucleus (step 10).

4 Apply an inclusive intensity threshold to the DQ-substrate channel.

Prior to measuring pixel intensities, most imaging programs require that the image data that constitutes signal be in some way distinguished from non-signal, or background, image data. This is commonly referred to as thresholding or segmentation and is usually applied on the basis of grayscale levels. Such a threshold defines the range of grayscale values that describe the regions of interest. Subsequent measurements are applied only to pixels that fall within this range. Although the selection is arbitrary, it must be held constant for all image stacks used for comparison. It is a good idea to record these threshold ranges for later reference. Use image stacks that represent the most intense and least intense signals when determining threshold range

- 5 Measure the intensities of all pixels under the threshold and log data to a spreadsheet.

Many advanced imaging software applications have several measures of intensity. It is best to measure integrated intensity rather than average intensity as this will incorporate the threshold range applied earlier. Some software packages output one integrated intensity value for each x,y image plane in the stack. In this case, sum this value across image planes and export data to a spreadsheet. This value will represent total degradation and later be averaged by the number of cells.

Separate intracellular from extracellular degradation

- 6 Combine the cell tracking and DNA-binding dye channels using an arithmetic addition function.

In order to completely mask all intracellular degradation, the fluorescence intensity data from the cell tracking and DNA-binding dye channels need to be combined.

- 7 Binarize the stack created in Step 6.

The fluorescence intensity data from the new stack need to be maximized or saturated at all pixels representing intracellular areas. This is done by converting the 8-bit image stack into a binary image stack. The result of the binerization is an image stack in which all pixels that contained cell tracking and DNA binding dye fluorescence are now at maximum intensity.

- 8 Determine pericellular degradation by applying an arithmetic subtraction of the binerized data obtained in Step 7 from the DQ-substrate channel image stack.

To calculate extracellular degradation (Deg_p), the fluorescence information (grey value) from areas considered intracellular must be removed using an image arithmetic subtract function. The binarized stack from step 7 (C) provides the spatial information (x,y coordinates) needed for the imaging software to subtract the grey value data from corresponding x,y coordinates in the total DQ-substrate channel stack (Degradation_T). The result of this function is the creation of a new stack (Degradation_p) containing only fluorescence data from extracellular regions. Measure and record the intensities of all pixels in the image stack.

$$\text{Degradation}_p = \text{Degradation}_T - C$$

- 9 Determine intracellular degradation by applying an arithmetic subtraction of the pericellular degradation stack created in step 8 from the total DQ-substrate channel stack (Degradation_T).

To calculate intracellular degradation, the fluorescence information (grey value) from the extracellular degradation stack (Degradation_p) must be subtracted from the total DQ-substrate channel (Degradation_T). The result of this operation is the creation of a new stack (Degradation_I) containing only fluorescence data from intracellular regions. Measure and record the intensities of all pixels of the image stack.

$$\text{Degradation}_I = \text{Degradation}_T - \text{Degradation}_p$$

Determine the cell number in image stack

- 10 Create a 3-D reconstruction using the fluorescence from the DNA-binding-dye channel.

Due to inherent differences in the sizes and shapes of cellular structures within an imaged area, degradation information (total, intracellular, pericellular) is normalized to the number of cells represented by the number of nuclei present. Accurate determination of nuclei requires the software to render nuclei in three dimensions and display a visual representation of the rendering that can be manipulated in three dimensions (x,y,z- directions) by the user.

- 11 Use a classifier to define all nuclei as objects and segment them from one another.

Most advanced image analysis software allows classification of objects based on intensity thresholds and size constraints. Many also have functions like noise reduction and separation of touching objects via an edge detection algorithm and shape constraints. Some software packages even allow classification of objects based on morphometric parameters, such as X and Y centroid, radial dispersion, texture moment difference, etc. These additional parameters are not generally necessary for the discrimination of ovoid objects such as nuclei from the background and from each other. Create a custom classifier combining as many classification criteria as needed to define nuclei as discrete objects in three dimensions. For the first image, it may be necessary to apply the classifier more than once, evaluating its ability to successfully describe the nuclei. When satisfied, save the classifier. It should apply to the other images as well, provided the same settings were used to acquire the images.

- 12 Count the objects.

If a counting option does not exist in the software, manually count the objects classified. Each measurement represents one object. Advanced imaging software will generally apply a unique color overlay to each identified object to assist the user in visualization of the distinct objects.

- 13 Evaluate the classifier in 3-D rendered mode.

Evaluate the classifier once again by rotating the 3D structure and viewing the overlay resulting from the measurement. If satisfied that the classifier has correctly segregated individual nuclei, record the count in the spreadsheet. Because this is highly subjective, more than one observer should evaluate.

- 14 Divide the desired intensity measure from the DQ-substrate channel by the total number of nuclei. [*CE:AQ: To obtain an activity per cell value?]

SUPPORT PROTOCOL 1

PREPARE CELL CULTURES ON ECM CONTAINING DQ-SUBSTRATES

The cell cultures being imaged must be grown on or within ECM containing the desired DQ-substrate. The ECM can exert a dramatically different effect on cellular morphology and physiology. The formation of unique structures such as monolayers, spheroids, acini, tubes, and branching clusters may depend on the composition and stiffness of ECM, media supplements, densities of cell seeding, etc. We have found that on gelatin and collagen type I cells primarily grow as 2-D monolayers like those seen on uncoated plastic and glass. In contrast, in complex matrices they form 3-D structures (e.g., see Fig. 4.20.2).

Prior to setting up cell cultures on ECM containing DQ-substrates, cells should first be prelabeled with a fluorescent cell tracking dye. Alternatively cells can be transfected or transduced to drive expression of a fluorescent protein such as monomeric Red Fluorescent Protein (mRFP), which we have found to remain cytosolic. There are two reasons to use fluorescently labeled cells: (1) to delineate the intracellular area taken up by cells in order to separate intracellular from extracellular degradation using imaging analysis software; and (2) to distinguish among the different cell types in co-cultures. Invitrogen provides several cell tracking fluorescent dyes with distinct excitation/emission spectra. This allows the user to label different cell types with unique colors, thus distinguishing each cell type in co-cultures. The investigator must be aware of the limitation of the microscope for handling multiple colors and availability of excitation and emission band-widths. After establishing cell cultures on ECM containing a DQ-substrate, the user can begin imaging immediately or at a later time. Cells will begin to degrade DQ-substrates shortly after seeding; however, sufficient hydrolysis for detection may require hours to accumulate. If long periods of culture time are required, as when the cells are slow to form 3-D structures or when degradation needs to be monitored in later stages of culture, the 3-D structures may need to be grown in the absence of DQ-substrates, harvested and then re-seeded onto coverslips coated with ECM containing DQ-substrate. Please see Support Protocol 2 for indications when reseeding should be considered.

Materials

Cell line of interest

CellTracker™ Orange CMTMR Dye (Invitrogen)

Cell culture medium, phenol red-free

DQ-substrates: DQ-collagen IV, DQ-collagen I, DQ-gelatin (Invitrogen)

Recombinant basement membrane (rBM): Matrigel (BD) or Cultrex (Trevigen)

Collagen I (Cohesion Laboratories)

Gelatin (Sigma)

Sucrose (Sigma)

12-mm no. 1 round glass coverslips, acid-washed and sterilized by baking

35-mm cell culture Petri dishes

1× and 10× phosphate-buffered saline, sterile (PBS; *APPENDIX 2A*)

0.1 M NaOH

- 1** Reconstitute DQ-substrate in water.

*All DQ-substrates are shipped from the manufacturer as a lyophilized powder requiring reconstitution. This is accomplished at room temperature with sterile distilled water for at least 1 hr. We recommend making a stock solution of 1 mg/ml. Once reconstituted, aliquots should be made and stored at 4°C. [*CE:AQ: What size aliquots?]*

- 2** Preload cells with membrane permeable cell tracking dye. [*CE:AQ: Please describe cell number/density, concentration of dye in what solvent, labeling conditions.]

Cells should be prelabeled 2–3 hr before establishing cultures on ECM containing DQ-substrate. The chemistries of the individual dyes differ and their distribution within the cell can vary among cell types. Invitrogen has developed

several thiol-reactive Cell Tracker™ probes that are non-toxic and yield fluorescent products that are retained inside live cells through several population doublings. These membrane permeable dyes undergo a GST-mediated reaction to produce membrane-impermeant glutathione–fluorescent dye adducts. We recommend that one test several dyes and determine concentrations and incubation times for each cell type to ensure dye distribution throughout cytoplasm. The duration of culture time before and during imaging must be considered since the dye is diluted as cells divide. We have found that Cell Tracker Orange CMTMR works well and will detail its use here (see below).

Dilute DQ-substrates in ECM

DQ-substrates need to be diluted in an appropriate ECM depending on your experimental requirements. There are several different commercially available ECM (gelatin, collagen I, rBM), which complement the different DQ-substrates. In our laboratory we dilute DQ-substrates with their constituent matched ECM (i.e., DQ-gelatin with gelatin, DQ-collagen I with collagen I, DQ-collagen IV with rBM). The rationale for diluting DQ-collagen IV in rBM is that collagen IV is a major component of basement membranes in vivo and in commercially available rBMs from EHS tumors. Each ECM requires slightly different handling techniques and therefore each will be discussed separately. Regardless of the DQ-substrate or the ECM used, we have found that the ideal final concentration of DQ-substrate is 25 µg/ml. Because there are lot-to-lot variations in both the DQ-substrates and the ECM, we recommend that all experiments be repeated with the same lot.

Prepare a gelatin + DQ-gelatin substrate

- 3a** Add 2% powdered bovine skin gelatin and 2% sucrose to sterile PBS.
- 4a** Heat to 56°C in a water bath until both gelatin and sucrose dissolve.
- 5a** Filter solution using a sterile 0.2-µm syringe filter.
- 6a** Allow to cool slightly before adding DQ-substrate at 25 µg/ml.
Avoid cooling to room temperature because gelatin will begin to solidify as it cools.

Prepare a collagen I + DQ-collagen I substrate

- 3a** Purchase collagen I and store at 4°C as a liquid.
- 4b** Prior to mixing with DQ-substrates, convert collagen I into a state in which it will solidify. To do this we make a solution containing 80% (w/v) collagen I, 10% (v/v) 10× PBS and 10% (v/v) 0.1 N NaOH (pH 7.4).
- 5b** At this point mix the collagen I solution with DQ-substrate to a final concentration of 25 µg/ml. Gently pipet to ensure thorough mixing.

Prepare recombinant basement membrane + DQ collagen IV substrate

- 3c** Thaw rBM should on ice overnight at 4°C.
The rBM remains a liquid on ice but solidifies rapidly when warmed so it should be handled on ice at all times.
- 4c** Dilute the appropriate amount of DQ-substrate with an appropriate amount of rBM in a prechilled container to a final concentration of

25 µg/ml. Mix on ice using gentle pipetting to avoid creating bubbles. Keep on ice.

Take extra precautions during pipetting as errors may occur due to the viscosity of the rBM.

Prepare cultures

- 7 Coat glass coverslips with ECM containing DQ-substrate. Place two round coverslips in a 35-mm cell culture dish. Using a P100 micropipettor, carefully pipet and spread 50 µl of the solution made above over the entire surface of each coverslip.

Take care not generate air bubbles and stay within the dimensions of the coverslip.

- 8 Promptly place in humidified incubator at 37°C without CO₂ and allow to solidify for 2 hr (gelatin), 15 min (rBM) or 30 min (collagen I).

The coated coverslips must be promptly placed in a humidified incubator to prevent dehydration/shrinking of the ECM. Include control coverslips that will be cultured without cells. Alternatively, the investigator may choose to directly coat the bottom of a 35-mm dish.

- 9 Seed cells onto coated coverslips While the rBM is solidifying, trypsinize and count the prelabeled cells (*UNIT 1.1*).

Be sure that cells are in a single-cell suspension.

- 10 Centrifuge the cells ?? min at 80–100×g, ??°C, and resuspend in culture medium so that the desired number of cells per coverslip is contained in a 50-µl volume.

If doing a co-culture experiment, a mixture of cell types should be made with the appropriate cell numbers mixed in a 50-µl volume of medium.

- 11 Place 50 µl suspension of cells onto each coated coverslips. Carefully place the 35-mm dish containing the cells on coverslips into a 37 °C incubator. Allow 30–60 min for the cells to attach to the ECM.

If performing co-culture keep in mind that one cell line may require longer times to adhere to the ECM.

- 12 Fill the 35-mm culture dish with 2 ml of culture medium. If using assay supplements, these should be added at this time.

- 13 Incubate for desired time before imaging.

The time period between seeding cells and imaging must be determined by the investigator and depends on the experimental design and cell types. Imaging can commence as soon as cells have attached to the ECM.

SUPPORT PROTOCOL 2

HARVESTING 3D SPHEROIDS OF CELLS GROWN IN RBM: AN ALTERNATIVE TO DIRECTLY GROWING ON ECM CONTAINING DQ-SUBSTRATES

When cells in rBM need to be grown over long periods of time before imaging, the cultures may first be grown without DQ-substrates and then transplanted into fresh ECM that has been mixed with the DQ-substrate. Harvesting the initial cultures from rBM requires gentle enzymatic digestion of the rBM so that the cellular structures can be removed with minimal disruption. Therefore, harvesting is only recommended for cell types that grow as tight

spheroids or aggregates. Harvesting will significantly alter the 3-D morphology of structures composed of tubes, networks and branches.

Materials

Cell culture grown on rBM within a 60-mm cell culture dish

Cell culture medium, phenol red-free

1× phosphate-buffered saline (PBS; *APPENDIX 2A*), sterile

6 U/mg lyophilized dispase (Roche)

- 1 Aspirate medium from the cell culture and wash cells twice with 5 ml PBS
- 2 Dilute the lyophilized dispase to 1.5 U/ml using sterile PBS. Add 2 ml dispase solution to completely cover the dish. Place culture dish into a 37°C incubator. After 15 min begin to periodically monitor dish with a standard microscope for the release of cellular structures.

Complete digestion should occur within 60 minutes.

- 3 Add 1 ml fresh PBS. Use gentle pipetting with 5-ml pipet to complete the release of cellular structures from the rBM and culture dish. Use additional PBS if needed. Collect cellular structures with pipet into a 15-ml conical tube.

- 4 Gently centrifuge structures 5 min at $80 \times g$, 22°C.

Spinning cells at $80 \times g$ for 5 min will be sufficient to pellet even the smallest clusters of cells. If structures are large (>200 cells), we recommend that structures be allowed to sediment to the bottom of a 15-ml conical tube instead of centrifuging.

- 5 Aspirate the PBS and resuspend structures in appropriate volume of fresh cell culture medium.

The volume used for resuspension will depend on the initial number of structures in the original dish and the number of structures desired per coverslip. As in Support Protocol 1, ~50 µl of media can be pipetted onto a coated coverslip

- 6 Gently pipet cellular structures onto freshly coated coverslips from Support Protocol 1 and incubate.

- 7 Image the cultures at the appropriate time.

COMMENTARY

Background Information

The ability of cells to degrade ECM had been imaged previously by growing cells on FITC-labeled ECM and looking for a loss in fluorescence as a result of ECM degradation. With this method it is often difficult to assess loss of fluorescence, in particular small losses, because the background fluorescence is so intense. Furthermore, this method does not assess proteolysis in real-time by live cells since the FITC-labeled matrices and cells are fixed prior to imaging. As a result the areas of fluorescence loss may not be associated with cells. For example, we have found tracks in which there is loss of fluorescence when tumor cells are grown on FITC-labeled ECM (Sloane 1996). These tracks suggest that the cells are migrating and degrading the ECM as they migrate. We developed the DQ-substrate assay described here so that proteolysis by live cells could be imaged in real-time.

The fluorescence of the DQ-substrates is quenched as a result of extensive labeling, the close association of FITC molecules and a transfer of non-radiative energy, i.e., a FRET effect. Upon proteolytic cleavage, the distance between the FITC molecules increases resulting in emission of fluorescence (see Fig. 4.20.1B and D). This increase in fluorescence occurs on a non-fluorescent background (see Fig. 4.29.1A and C) and thus is readily observed. Furthermore, the gain in fluorescence can be imaged without fixation. When we use DQ-substrates for imaging of proteolysis by live cells, we see fluorescent fragments of DQ-substrates both extracellularly and intracellularly. At least some of the intracellular fluorescence represents intracellular degradation as cell-permeable inhibitors of lysosomal cysteine cathepsins reduce the amount of intracellular fluorescence (Sameni et al. 2003; Sameni et al. 2000). The presence intracellularly of fluorescent fragments of DQ-substrates requires endocytosis by live cells. This may be due to endocytosis of intact DQ-substrate and its degradation intracellularly or endocytosis of fluorescent fragments of DQ-substrate that had been degraded extracellularly. In either case, the intracellular fluorescence could not be visualized unless the cells were alive, a clear advantage of the DQ-substrate assay. A potential disadvantage is that the DQ-substrates are proteins so they do not allow one to directly assess the activity of any individual protease or protease class. As some DQ-substrates are proteins that would be encountered in vivo by migrating or invading cells, e.g., collagen IV or I, analyzing their degradation could allow one to identify proteases that are involved in normal developmental or pathological processes. Furthermore, more than one protease or protease class can degrade these protein substrates and thus their use should allow one to identify multiple proteases and potentially proteolytic pathways that are involved in these processes.

Another advantage of DQ-collagen substrates is that they allow one to study proteolysis in cells growing in a 3-D context that mimics the in vivo environment. There are several excellent reviews on the value of 3-D in vitro models (Schmeichel and Bissell, 2003; Debnath and Brugge, 2005; Yamada and Cukierman, 2007)

Critical Parameters

This protocol describes methods to set up and image cell cultures using an upright microscope equipped with long-working-distance dipping objectives. Although an upright microscope is ideal for the sample thicknesses of 3-D cultures, inverted microscopes can be used. Several microscope companies manufacture long-working-distance (2–3 mm) high-power dry objectives such as the Zeiss 40× LD and 63× LD Plan-neofluar, which can be used in inverted microscopes. Along with long-working-distance dry objectives, growing cells on glass-bottom culture dishes (coverslip thickness) will minimize the distance between the objective and the cells within the ECM. Furthermore, microscope-grade glass has optical characteristics that are optimal for high-quality imaging.

Troubleshooting

Fluorescence from DQ-substrates appears as large, amorphous deposits that are not associated with cells. The DQ-substrates may have been handled improperly following solubilization. Once in solution the DQ-substrates should not be frozen. Do not vortex any solution containing DQ-substrates and avoid excessive pipetting. Also do not let either the substrate or the ECM dry out. Make sure that the lot number of DQ-substrate has not expired or been recalled.

Collagen I does not adhere well to glass. We recommend establishing cell cultures on collagen I polymerized within a 35-mm plastic culture dish. Note that the increased surface area will also mean that higher cell numbers must be seeded.

Anticipated Results

Specific results will depend on the cells analyzed, the use of monolayer or three-dimensional cultures, and the DQ-substrates and ECM used for the assay. Degradation of the DQ-substrates can be seen within hours after plating and can be imaged in real-time in cultures growing in an incubator on the microscope stage. If stability of the set-up can be maintained, cultures can be imaged in real-time for long time periods; we have imaged for as long as 24 hr (Cavallo-Medved et al., submitted). This requires a stage incubator that can maintain temperature, humidity, oxygen tension, and CO₂. Degradation of the DQ-substrates is seen within hours after plating. Not surprisingly, the denatured substrate DQ-gelatin is degraded faster than is either DQ-collagen I or DQ-collagen IV. Whether the DQ-substrates are degraded extracellularly, intracellularly or both may depend on the ECM in which they are mixed. The ECM can affect the morphology of the cells and whether they grow as two-dimensional monolayers or three-dimensional structures (e.g., see Fig. 4.20.2) and in addition can influence the ability of the DQ-substrates to be endocytosized.

Time Considerations

3D cellular structures and cocultures require long imaging times. The thicker a 3-D structure is the more optical sections needed to image. It may take minutes to record an optical section into each fluorescent channel; this is particularly true for high-resolution images requiring long scan times. If one is imaging cocultures in which each cell type is labeled with a fluorescent dye/marker, then each color must be scanned into a separate fluorescent channel. Another consideration is that variability among structures requires imaging of a greater number of structures if the data are to be statistically significant.

Time at which one should perform fluorescent labeling. Since cytoplasmic dyes that can be used to prelabel cells will be diluted out with each cell division, these dyes can only be used to label short-term cultures or need to be added shortly before imaging, e.g., 2 hr (Support Protocol 1). The timing of staining with DNA binding dyes such as Hoechst 33342 may affect cell viability. Due to their interaction with DNA, these dyes will induce apoptosis of most cells within 12 hr.

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Figure 4.20.1.

Culture with live cells is required to cause release of green fluorescence from DQ-substrates. Confocal images of green fluorescent channel at a z-level below the ECM-medium border. Cultures containing either collagen I with DQ-collagen I (**A,B**) or rBM containing DQ-collagen IV (**C,D**) were imaged after 3 days of culture without cells (**A,C**), with human breast cancer cells (**B**) or with human breast epithelial cells (**D**). Bar, 50 μ m.

Figure 4.20.2.

Examples of cellular morphologies observed on different ECM. Phase contrast or DIC images of: (A) human breast cancer cells forming a simple monolayer on gelatin; (B) human prostate cancer cells forming clusters and sheets on collagen type I; (C) human colon carcinoma cells forming a 3-D spheroid of polarized cells surrounding a hollow lumen in rBM; and (D) human umbilical vein endothelial cells forming a tubular network of cellular cords on rBM.

Figure 4.20.3.

Frame captures of 3-D reconstructions of human breast cancer cells grown in rBM containing DQ-collagen IV. Image stacks containing the DQ-substrate channel (green), the nuclei channel (blue) and the CellTracker channel (red) were used to create graphical 3-D reconstructions/projections using the QVTR feature of the Volocity imaging software suite. The 3-D image can be rotated 360° in order to (A) view proteolysis of DQ-substrates (green) in relation to the cells (red) or (B) view only the nuclei for the purpose of counting cells.

Figure 4.20.4.

Quantification of proteolysis and discrimination of intracellular and extracellular localization of degradation fragments in human breast epithelial cells grown in rBM containing DQ-collagen IV. (A) Single optical section at equatorial plane showing fluorescence of the CellTracker Orange channel. (B) Same optical section but a binarized image of the CellTracker Orange and DNA dye channels. (C) Total (extracellular and intracellular) degradation fragments of DQ-collagen IV in same optical section. (D) Extracellular degradation fragments of DQ-collagen IV, obtained by masking image of panel C with the image from panel B to eliminate all signal from intracellular areas. (E)

Intracellular degradation fragments of DQ-collagen IV. **(F)** Total degradation fragments of DQ-collagen IV in all optical sections were quantified as normalized integrated intensity per cell. Total fluorescence separated into intracellular and pericellular components using image arithmetic in MetaMorph software.