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Imaging Podosome Dynamics and Matrix Degradation

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Summary

Invasive cell migration is critical for leukocyte trafficking into tissues. Podosomes are matrix-degrading adhesive structures that are formed by macrophages and are necessary for macrophage migration and invasion. Here, we describe methods for imaging and quantifying podosomes in primary human macrophages and in THP-1 cells, a monocyte cell line that can be differentiated to a macrophage-like state. Moreover, we outline detailed methods for live imaging of podosomes, which are highly dynamic, and for the quantification of rates of podosome turnover. Finally, we discuss methods for the quantitative analysis of matrix degradation on fluorescent-gelatin coated cover slips.

Keywords

podosomes; podosome turnover; podosome dynamics; macrophage; THP-1; live imaging; gelatin degradation; Lifeact; invadopodia

1. Introduction

Cell migration and tissue invasion are important functions in both physiological and pathological processes. Some highly motile and invasive cells form podosomes and invadopodia, which are structures uniquely adapted to these functions. Podosomes are the characteristic adhesive structures of monocyte-lineage cells but are also formed in Src transformed fibroblasts (1) and smooth muscle cells (2, 3), while invadopodia are protrusive structures primarily formed by invasive cancer cells (Figure 1) (4). Both podosomes and invadopodia share the ability to protrude into extracellular matrix and degrade it with proteolytic enzymes (reviewed in (4)). With half-lives of generally less than 5 minutes, podosomes are able to rapidly alter their organization, which is presumably necessary for migrating leukocytes to respond to inflammatory stimuli (4, 5).

Podosomes are easily distinguished from other types of adhesions due to their characteristic dot and ring morphology (Figure 1a-b). In addition to the morphology of individual

podosomes, another striking feature of podosomes is the ability to form highly organized rings and rosettes containing tens to hundreds of individual podosomes (4, 6). The dot at the core of the podosome is predominantly composed of F-actin and F-actin regulatory proteins. Wiskott-Aldrich Syndrome Protein (WASP)/N-WASP, cortactin, and Arp2/3 are several components of podosome cores that are shared with invadopodia (Table 1) (Figure 2) (7). The podosome ring structure is formed by integrins and integrin-associated proteins, including vinculin, talin and paxillin (Figure 2) (8). Interestingly, these and many other proteins are shared by both podosomes and focal adhesions (Figure 1c) (Table 2). The presence of these focal adhesion-associated proteins in podosomes may help explain the observation that podosomes play a more significant role in substratum adhesion than invadopodia (4, 9).

In response to inflammatory stimuli, the effector functions of podosomes are needed at multiple stages as monocytes traffic from the blood and as macrophages and dendritic cells migrate through tissues. The processes of endothelial transmigration, invasion through the basement membrane, and chemotaxis to sites of inflammation all appear to require podosomes. The initial steps of endothelial transmigration, classically described as rolling, activation, and arrest, are well defined (reviewed in (10)). During the process of endothelial transmigration, an early step in the inflammatory response, evidence suggests an important role for podosomes. When lymphocytes and monocytes are added to a monolayer of endothelial cells, podosome-like structures create invaginations in endothelial cells that coalesce into pores, thereby facilitating transcellular diapedesis (11). Reaching a site of inflammation also requires invasive migration through the vascular basement membrane and connective tissue. Macrophages from mice lacking Hck, a Src-family kinase in myeloid cells, form fewer podosomes and fail to accumulate in the peritoneal cavity after thioglycollate-induced peritonitis, indicating that podosomes may be playing a role in macrophage invasion (12). Furthermore, podosomes are known to form near the leading edge of chemotaxing cells (8, 13). The loss or mutation of proteins that regulate podosome formation, such as WASP (13, 14), verprolins (15), and ARF6 (16), also results in dysfunctional chemotaxis. While the evidence that podosomes play a role in these functions is not yet definitive, diseases in which podosome function is altered provide additional support for their physiological importance.

Diseases that result in aberrant podosome function highlight the essential role of podosomes for in vivo immune responses. Macrophages from patients with disease-associated mutations in WASP lack podosomes and have revealed a key role for WASP in both podosome formation and dynamics (6, 17). The classic presentation of Wiskott-Aldrich Syndrome is the triad of thrombocytopenia, eczema, and immune deficiency. Additionally, these patients are predisposed to autoimmunity (17). Because of the key role of WASP in regulating podosome dynamics (7), matrix degradation (14), and chemotaxis (13, 15), altered podosome function is likely an important component of the immune deficiency experienced by these patients. Macrophages from patients with the autoinflammatory disease, pyogenic sterile arthritis, pyoderma gangrenosum, and acne (PAPA) syndrome, also exhibit impaired podosome formation and decreased invasive potential (18). While the associations of disease with altered podosome function are correlative, there seems to be a substantial link between podosomes and appropriately regulated immune responses. There is, therefore, considerable

interest in understanding the mechanisms that regulate podosome formation and turnover. Here we present methods for studying the function of podosomes—live imaging of podosome dynamics and quantitative analysis of podosome-mediated gelatin degradation.

2. Materials

2.1 Blood Collection and Differentiation of Monocyte-Derived Macrophages

1. Phlebotomy Equipment: 30 mL syringes, alcohol prep pads, tourniquets, 2 × 2 inch sterile gauze pads, bandages, and a sharps container. We find that a Becton Dickinson Vacutainer® Safety-Lok™ Blood Collection Set with the following specifications is best for our needs: 21G 3/4 inch needle with 12 inch tubing and female luer. However, many functional equivalents are available.
2. Heparin sodium from porcine intestinal mucosa 1,000 USP units/mL (Abraxis Pharmaceutical Products, Cat. #63323-540-11) is stored at room temperature.
3. Phosphate buffered saline (without Ca²⁺ or Mg²⁺). All PBS used in this protocol is without Ca²⁺ or Mg²⁺.
4. Lymphoprep™ (Axis-Shield) is stored at room temperature. It must be kept in the dark due to light sensitivity.
5. 10 cm tissue culture-treated plates.
6. Macrophage medium. Based on RPMI-1640 plus the following additional components: 10% (v/v) fetal bovine serum (FBS), 0.15% (w/v) NaHCO₃, 0.45% (w/v) glucose, 10 mM HEPES, 1 mM sodium pyruvate, 100 IU/mL of penicillin, 100 µg/mL of streptomycin, and 0.05 mM β-mercaptoethanol. The FBS used for macrophage medium is not heat inactivated.
7. Recombinant human macrophage colony stimulating factor (rhM-CSF) (R & D Systems) is diluted with PBS to 20 µg/mL and stored in 31 µL aliquots at −20° C. After thawing on the day of blood collection, it is stored at 4° C during the subsequent week when it will be used for the differentiation of macrophages.

2.2 Culture and Differentiation of THP-1

1. THP-1 cells. This monocyte cell line is available from the ATCC (ATCC# TIB-202).
2. THP-1 medium. This medium is based on RPMI-1640 with the following additions: 10% heat-inactivated fetal bovine serum, 0.05 mM β-mercaptoethanol, 100 IU/mL of penicillin, and 100 µg/mL of streptomycin.
3. Phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, Cat. #P8139). A stock solution is made by dissolving the powder/film to a concentration of 2 mg/mL in 100% ethanol. The stock is stored in small aliquots at −20° C for several months.
4. THP-1 Imaging Medium. Ham's F-12 medium (without phenol red) with 10% heat-inactivated fetal bovine serum, 10 mM HEPES, and 0.05 mM β-mercaptoethanol.

2.3 Lentiviral Transduction of THP-1

1. Lentiviral particles encoding shRNA targeted against a gene of interest.
2. 4 mg/mL polybrene.

2.4 Nucleofection of Primary Macrophages

1. Nucleofector® II Device (Lonza).
2. Human Macrophage Nucleofector® Kit (Lonza). Prior to the use of the kit, mix the supplement with the main vial of nucleofector solution. Store nucleofector solution at 4° C. This kit includes the cuvettes and transfer pipettes required for the procedure, as well as an aliquot of the pMaxGFP® vector at 0.5 µg/µL.
3. pRuby-Lifeact at 1 µg/µL.
4. pEGFP-vinculin at 1 µg/µL.
5. 1X TRED (50 mL): 0.25% trypsin and 0.02% (w/v) EDTA in PBS (without Ca²⁺ or Mg²⁺).
6. Gel loading pipet tips.
7. Glass-bottom 35 mm tissue culture dishes (*see* Notes 1 and 2).
8. 10 µg/mL fibrinogen. 10 mg/mL fibrinogen stock solution is prepared by solubilizing powdered human fibrinogen (Sigma, Cat. #F4883) in ddH₂O. It is stored in single use aliquots at –80° C. For coating glass bottom dishes the stock solution is diluted to 10 µg/mL in PBS. 250 µL is needed per dish. 10 µg/mL fibronectin may be substituted. We purify fibronectin from human plasma as previously described. (19)

2.5 Live Imaging of Podosomes

1. Macrophage Imaging Medium. Made with Ham's F-12 medium (without phenol red) and the following additions: 10% fetal bovine serum (FBS), 0.15% (w/v) NaHCO₃, 0.45% (w/v) glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 0.05 mM β-mercaptoethanol. (*see* Note 3)
2. Inverted epifluorescence or confocal microscope with a 60X-100X objective, filter wheels with excitation and emission filters appropriate for imaging EGFP and

¹Glass bottom dishes: The dishes may be purchased commercially, but they are relatively easy to make in house. Drill a 16–18 mm hole in the center of the bottom half of a standard 35 mm tissue culture dish. Invert the dish so that the outside surface is facing up. Place a thin bead of Norland Optical Adhesive 68 around the hole. Place an acid-washed 22 mm cover slip (*see* Note 2) on the bead of glue and press down lightly. This glue is cured by UV light. Approximately 1 hour in a UV transilluminator or overnight in the UV light of a tissue culture hood is sufficient to cure the glue. Sterilize by rinsing with 70% ethanol and exposing to a UV light source.

²Acid-washed cover slips: In a 500 mL beaker on ice (this solution gets hot), make 200 mL of 3.6 M H₂SO₄. When the solution has cooled to room temperature, add the desired amount of cover slips and stir to ensure that all cover slips are exposed to the acid. Cover and incubate overnight. The next morning, pour as much of the acid as possible into a waste beaker and neutralize appropriately. Neutralize the beaker of cover slips by adding 200 mL of 0.1 M NaOH. Adjust the pH of the cover slips to between 6 and 8. Discard this solution. Rinse the cover slips 2 times with ddH₂O. Dry the cover slips in a single layer on Whatman paper. These can be stored for several months.

³There are two especially important components of the imaging medium that deserve mention. It is necessary to use medium without phenol red in order to reduce background fluorescence. The addition of HEPES maintains the neutral pH of the medium in an environment with atmospheric levels of CO₂.

mRuby/RFP, a camera, and a shutter. The filters, shutter, and camera should be computer-controlled. The microscope should be contained in an environmental chamber, which can be maintained at 37° C by a temperature control unit.

3. Microscope control and image editing software. We use Metamorph® software (Molecular Devices) for integrated microscope control and image analysis. However, other software capable of performing similar functions is also appropriate.

2.7 Production of Gelatin-Coated Cover Slips

1. 70% (v/v) ethanol (EtOH).
2. 50 µg/mL poly-L-lysine (PLL): 1 part PBS and 1 part 0.01% (100 µg/mL) PLL. Make 150 µL per cover slip on the day of use.
3. 0.5% glutaraldehyde: Dilute 25% glutaraldehyde stock solution 1:50 in PBS on the day of use. Make 150 µL per cover slip.
4. 200 µg/mL green gelatin: Make a 2 mg/mL stock by dissolving Oregon Green® 488-conjugated gelatin from pig skin (Invitrogen) in PBS. Make a 200 µg/mL working solution by diluting the stock 1:10 in PBS, 50 µL per cover slip. Extra stock solution can be stored at –20° C for future use.
5. 5 mg/mL NaBH₄: Make 150 µL per cover slip by dissolving powdered NaBH₄ in PBS at the time specified in the protocol. Allow the tube to vent after the solution is made.

2.8 Imaging Podosome-Mediated Gelatin Degradation

1. Acid Washed 10 mm cover slips (*see* Note 2).
2. Fixative: 4% paraformaldehyde and 0.25 mg/mL saponin in PBS. This should be made fresh on the day of use, 500 µL per cover slip.
3. Quenching Solution: 150 mM glycine in PBS, 500 µL per cover slip.
4. Blocking Buffer: 10% goat serum (alternatively, heat inactivated FBS) and 0.25 mg/mL saponin in PBS. This should be made on the day of use, 600 µL per cover slip.
5. 200 units/mL rhodamine phalloidin (Invitrogen).
6. DAPI: Dissolve in ddH₂O to make a 20 mg/mL stock and store in small aliquots at –80° C. Dilute stock 1:2000 in ddH₂O for staining, 100 µL per cover slip.
7. Accutase cell detachment solution (Innovative Cell Technologies) is needed if THP-1 cells are being used. Store aliquots at –20°C. Once thawed, the solution is stable at 4°C for several weeks.
8. Mounting Medium (*see* Note 4).

3. Methods

3.1 Blood Collection and Differentiation of Monocyte-Derived Macrophages

This section will describe the methods used to obtain blood, isolate mononuclear cells, and differentiate monocytes into macrophages. As a general guideline, 30 mL of blood will yield one 80–100% confluent 10 cm plate of macrophages ($1-3 \times 10^6$ cells). We often collect 60 mL to ensure that enough cells are available for multiple nucleofection conditions. This protocol assumes that proper IRB approval for human blood collection has been obtained.

1. Assemble all supplies needed for blood collection before beginning the procedure.
2. Using a fine gauge needle and 1 mL syringe, add heparin to the syringe being used for the draw so that the final concentration will be 10 U/mL after the desired volume of blood is collected (i.e. use 300 μ L of 1000 U/mL heparin for a 30 mL collection). Express any remaining air from the syringe and attach the Blood Collection Set. Alternatively, heparinized vacutainer® tubes and holders may be used, but this requires multiple tube changes for the volumes being collected.
3. Collect 30–60 mL of venous blood according to standard practices.
4. Working in a sterile tissue culture hood, empty the blood from the syringe into a sterile flask or beaker. Dilute the blood 1:1 in PBS (without Ca^{2+} or Mg^{2+}).
5. Add 15 mL of Lymphoprep™ to each of two 50 mL conical tubes. Gently overlay 30 mL of the diluted blood on the Lymphoprep™. Do not allow the layers to mix.
6. All of the subsequently described spins for this prep require a swinging bucket centrifuge and are performed at 25°C without the brake. Spin the samples for 30 minutes at 500 x g. After this first spin, the following four layers will be present from top to bottom: a straw colored plasma layer, the white “buffy coat” containing leukocytes, the clear lymphoprep, and the red blood cells.
7. Collect the buffy coats from both tubes as completely as possible and pool them in a fresh 50 mL conical tube. Add PBS to a final volume of 50 mL and spin at 500 x g for 15 minutes. Gently aspirate and discard the supernatant, which may be opaque after this first spin. The pellets are relatively soft after this and the subsequent spins. Take care not to disturb them.
8. Resuspend the pellet in 25 mL of room temperature PBS and spin at 330 x g for 12 minutes.

⁴We make our own mounting medium, which utilizes phenylenediamine as an anti-fading agent. This recipe makes approximately 25 mL. Weigh 6.0 g of ultrapure glycerol into a 50 mL conical tube. Weigh 2.4 g of polyvinyl alcohol and add it to the tube of glycerol. Stir this mixture with a glass rod before adding 6 mL of sterile ddH₂O. Incubate 4 hours to overnight at room temperature. Make 12 mL of 0.1 M Tris base pH 8.5 and add 10 mL of this solution to the mixture. Place the beaker in a 50°C water bath and stir for 10 minutes. After 6 minutes of stirring, weigh 30 mg of phenylenediamine and add it to the remaining 2 mL of 0.1 M Tris. It is very important to work quickly after the phenylenediamine is in solution, as it oxidizes rapidly. Add the phenylenediamine solution to the 50 mL conical and stir with a glass rod until a uniform solution has been created. Cool for 3 minutes on ice and spin the mounting medium down in a 4°C swinging bucket centrifuge for 15 minutes at 2000 x g. Make 0.5–1 mL aliquots and store at –80°C until use. When using the mounting medium, it is important to thaw it on ice and return it to –80°C as soon as possible to slow oxidation. When fresh, the mounting medium is pale brown, and it should be discarded if it becomes a deeper brown.

9. Resuspend the pellet in 25 mL of macrophage medium pre-warmed to 37°C and spin at 330 x g for 12 minutes.
10. After this final spin, aspirate the supernatant and resuspend the pellet in 10 mL of macrophage medium. Add to a 10 cm tissue culture-treated dish and incubate at 37°C with 5% CO₂ for approximately 4 hours to allow the monocytes to adhere to the plate (*see* Note 5).
11. Remove the lymphocyte fraction from the prep by swirling the plate *very gently* to resuspend any non-adherent cells that have settled to the bottom of the dish, and aspirate the medium. The monocytes are only lightly adherent at this point, and it is important not to disturb them. Adding and removing medium from a single location on the plate will further minimize disruption.
12. Add 10 mL of warmed macrophage medium containing 20 ng/mL rhM-CSF. On days 3 and 5 after isolation, the medium should again be aspirated from the macrophages and replaced with 10 mL of fresh medium plus rhM-CSF. The macrophages will be fully differentiated and ready for use on days 6–7 after the initial isolation.

3.2 Culture and Differentiation of THP-1

THP-1 cells are an accepted model system for the study of macrophage function (20, 21). Here, we describe the culture requirements of THP-1 cells and the use of PMA to differentiate them to a macrophage-like state. After differentiation, they can be used in place of primary macrophages for live imaging or gelatin degradation with some modifications of the described protocols. If THP-1 cells are used for nucleofection and live imaging, then they should be differentiated after nucleofection.

1. THP-1 are maintained in THP-1 medium at 37°C with 5% CO₂. Cultures are maintained according to ATCC recommendations, keeping cells within the range of 2×10^5 to 1×10^6 cells/mL. They are generally subcultured to a density of 2×10^5 after reaching $7\text{--}9 \times 10^5$ cells/mL.
2. In order to differentiate the cells to a macrophage-like state, spin down an aliquot of cells for 4 minutes at 500 x g. Resuspend the cells at a density of 5×10^5 cells/mL in THP-1 medium plus 500 nM PMA. Add the cells to a 10 cm suspension plate (smaller if necessary) and return to a 37°C with 5% CO₂ incubator for 3 hours. Although a suspension plate is preferred, a tissue culture-treated plate will work, but lifting the cells requires more pipetting. This 3-hour treatment with PMA initiates the differentiation process, but it is necessary to wait 48–72 hours after treatment before using the cells for imaging, as differentiation continues in the absence of PMA.

⁵When placed in a tissue culture-treated dish, the lymphocytes remain in suspension while the monocytes and platelets adhere. During the course of the 3 medium changes over the subsequent 5 days, the vast majority of the remaining lymphocytes are removed. During this time, many platelets will be removed, but they will continue to detach over the course of the 7 days of differentiation. By the time the cells are used for experiments, they are a relatively pure population of macrophages.

3. After 3 hours, the cells are lightly adherent. Lift them by pipetting over the entire plate. Pellet the cells by spinning for 4 minutes at 500 x g. Wash once in a volume of THP-1 medium at least as large as the volume used for differentiation. Pellet the cells as described.
4. Resuspend the cells at a concentration that is appropriate for the intended use in THP-1 medium without PMA. This will be described in subsequent steps of the protocol.

3.3 Lentiviral Transduction of THP-1

Manipulation of endogenous gene expression is a valuable tool for studying many cellular processes. shRNA-mediated knockdowns have been used to study the role of several proteins in podosome function (14, 22). Lentiviruses are a convenient vehicle for shRNA delivery because of their high transduction efficiency (23). Ready-to-use lentiviral particles are available from multiple companies. Alternatively, several systems are available for producing shRNA-encoding viruses in the lab. Here, we describe an infection protocol that we have used to successfully knock down target genes in THP-1 cells.

It is important to note that while lentiviral vector systems are generally replication incompetent, viruses with tropism for human cells can infect lab workers. Caution should be taken during the construction of lentiviruses to ensure that genes with significant potential to damage humans, such as oncogenes, will not be expressed. Furthermore, personal protective equipment should be used when handling lentiviruses, and proper decontamination procedures should always be followed after handling them (Safety precautions reviewed in (24)).

1. On the day before infection, split a culture of THP-1 cells to a density of 4×10^5 cells/mL. 10^6 cells will be needed for each knockdown condition.
2. On the day of infection, spin down aliquots of 10^6 THP-1 cells for 4 minutes at 500 x g. At a minimum, the following samples and controls should be used:
 - a. Lentivirus targeting the gene(s) of interest. Typically, approximately 3 shRNA targets are tested for each gene in order to identify an optimal knockdown.
 - b. Lentivirus encoding a scrambled or off-target control
 - c. An uninfected sample
3. Mix the viral particles to be used for each infection in 4 mL of THP-1 medium containing 4 µg/mL polybrene.
4. Resuspend the pelleted THP-1 cells in the appropriate virus-containing medium. Add the cells to a 6 well plate.
5. Spin the cells on a microplate carrier in a 32°C swinging bucket centrifuge for 1.5 hours at 400 x g.
6. Return the cells to a 37°C, 5% CO₂ incubator for 3 days to maximize infection prior to beginning selection.

7. Common selection mechanisms for lentiviral systems are puromycin resistance and fluorescent protein (ie GFP) expression. If a negative selection system, such as puromycin resistance, is being used, transduced cells should be selected for 7–14 days in selection medium. When all of the uninfected control cells have died and the transduced cells are growing rapidly in selection medium, selection is complete. The cells may be kept in selection medium indefinitely, but we have found that fully selected lines reliably maintain their knockdowns when cultured in standard THP-1 medium. If your system uses a positive selection marker, such as GFP expression, then transduced cells can be separated from non-transduced cells using fluorescence-activated cell sorting (FACS).
8. The knockdown should be confirmed with western blot analysis. Knockdown lines can then be used in the following protocols to study podosome function.

3.4 Nucleofection of Primary Macrophages

Nucleofection is a proprietary transfection method that utilizes solutions and electrical impulses that have been developed for individual cell types. It is useful for expressing constructs in cells that are otherwise difficult to transfect, such as terminally differentiated primary macrophages (25). In this protocol a Lifeact-mRuby fusion is nucleofected into macrophages to monitor the podosome cores. Lifeact is a 17aa actin probe derived from the yeast protein, Abp140, which is reported to have minimal effects on actin dynamics due to a relatively low binding affinity (26). Vinculin-GFP is used to study the behavior of the podosome rings. It is also possible to nucleofect THP-1 cells for use in live imaging. We use a THP-1 nucleofection protocol described by Schnoor et al. before treating the cells with PMA to induce differentiation (27). After a 3 hour PMA treatment, they can be added to glass bottom dishes and imaged after 2 days of differentiation.

1. Before preparing cells for nucleofection, it is best to have all of the necessary reagents at hand due to the toxicity of the nucleofector solution if exposure is prolonged.
2. Approximately 1 hour before nucleofecting the cells, coat the glass bottom dishes being used with 10 µg/mL fibrinogen. Alternatively, 10 µg/mL fibronectin may be used. Add 250 µL of 10 µg/mL fibrinogen to the glass area of the dish only. Incubate at 37°C for 1 hour, and wash the plates twice with 2 mL of PBS. The second wash may remain in the plate until the macrophages are ready to be added.
3. At least 1 hour prior to the actual nucleofection, place 10 mL of macrophage medium in a dish at 37°C with 5% CO₂ to charge.
4. In 500 µL eppi tubes, mix 100 µL of Human Macrophage Nucleofector Solution with each of the following plasmids (*see* Note 6):
 - a. 0.5 µg of pRuby-Lifeact plus 0.5 µg of pEGFP-vinculin

⁶The pMax-GFP nucleofection is included so that the nucleofection procedure may be optimized in reference to other published protocols. These tend to define transfection efficiency by the percentage of pMax-GFP expressing cells. The 'No DNA' nucleofection is included as a control for the toxicity of the nucleofection solution. Additionally, macrophages are auto-fluorescent at a wavelength that is visible in the GFP channel, and it is good to familiarize yourself with this background in order to evaluate real vs. auto-fluorescent signal.

b. 1 μ g of pMaxGFP

c. No DNA

- 5.** These solutions should reach room temperature before use in nucleofection. Leave the stock vial of nucleofector solution at room temperature until all nucleofections are completed in case extra is needed (*see* Note 7).
- 6.** Turn on the Nucleofector device and enter program code Y-010.
- 7.** Aspirate medium from the plate of macrophages and wash 2×5 mL with warm PBS. Add 2 mL of warm TRED and return the plate to the incubator for 5–7 minutes. Remove plate from the incubator and pipet vigorously over the entire plate using a P1000 to lift the cells (*see* Note 8). Confirm that all cells have been lifted under the microscope, and transfer the cells to a 15 mL conical containing 6 mL of warm macrophage medium. Add 2 mL of macrophage medium to the plate to wash off any remaining cells, and add this to the conical (final volume $\approx$ 10 mL).
- 8.** Perform a rough count of the cells. Expect to have $1\text{--}3 \times 10^6$ macrophages in total. Pellet the cells for 4 minutes at $500 \times g$, preferably in a swinging bucket centrifuge.
- 9.** Thoroughly resuspend the cells in a volume of PBS that would result in a concentration of $\sim 1 \times 10^6$ cells/mL according to the previous count.
- 10.** Perform an accurate count and aliquot 5×10^5 cells into each of 4 eppi tubes (1 extra in case of nucleofection problems), and spin down for 4 minutes at $500 \times g$.
- 11.** Completely aspirate the supernatant and resuspend in 100 μ L of the appropriate nucleofector solution/DNA mixture. Resuspend gently, being certain to avoid the formation of bubbles. After adding nucleofector solution to the cells, work as quickly as possible until it has been neutralized post-nucleofection.
- 12.** Add the contents of each of these tubes to nucleofector cuvettes using gel-loading pipet tips. Again, be sure not to introduce bubbles. If the solution is not spread evenly across the bottom of the cuvette, tap it lightly on the work surface to redistribute the liquid.
- 13.** Nucleofect all of the samples, using the Y-010 program.
- 14.** As soon as possible after nucleofection, add 1 mL of the charged macrophage medium to each cuvette, allowing any debris to float to the surface. Use the transfer pipets supplied with the nucleofector kit to remove all but the top-most 50–100 μ L from the cuvette to reduce collection of debris. This volume should contain enough cells to split between 2 glass bottom dishes. Add cells to the glass bottom dishes so

⁷Despite the precautionary measures that we recommend in this protocol, we still find that nucleofections occasionally do not work. In these cases, the nucleofector device will report an error, and cells in that sample will no longer be viable. For this reason we recommend having extra cells, DNA, and nucleofector solution ready until all of the desired conditions are completed.

⁸Macrophages are strongly adherent and difficult to lift. In order to increase viability, keeping the cells in TRED for the minimum amount of time is important. After incubating the cells at 37°C for 5–7 minutes, we find that the cells can be lifted efficiently by pipetting with a P1000. Generally, pipetting 10–15 times across the plate while holding in one orientation, turning the plate 90°, and repeating the washing will lift all of the macrophages.

that the medium is pooled in the center of the dish, concentrating the cells in the area to be imaged.

15. Allow the cells to adhere for approximately 4 hours. Gently aspirate the medium, wash the plate with 2 mL of macrophage medium to remove debris, and add 2 mL of fresh medium, this time filling the entire dish.
16. Allow cells to recover and begin expressing the transfected constructs for at least 12 hours prior to imaging. Proceed with live imaging as described in section 3.5.

3.5 Live Imaging of Podosomes

Podosomes are highly dynamic structures with half-lives generally less than 5 minutes (4, 5). Manipulations that alter podosome stability also perturb normal podosome function, demonstrating the important link between the two. For example, inhibition of calpains, intracellular proteases that cleave many cytoskeletal proteins, results in stabilized podosomes due to decreased disassembly (28). Increased podosome stability correlated with decreased random migration and transmigration across an endothelial monolayer (28). Conversely, cells that expressed a phospho-mimetic WASP mutant formed more dynamic podosomes and degraded gelatin to a greater extent than their wild type counterparts (14). The following methods describe the techniques required for live fluorescence imaging of podosomes and the subsequent analysis of their turnover (Figure 3). The method for quantification of podosome turnover was developed by Webb and Horwitz, and modified by our lab to quantify the turnover of multiple adhesion types (29, 30). The rate constants of podosome assembly and disassembly are determined as functions of the change in fluorescence intensity over time. It is important to note that the method can only be used to measure turnover of the podosome cores, as the rings are typically too close together to isolate.

1. At least 2 hours (preferably overnight) before the beginning of imaging, turn on the temperature control unit. This will allow all components of the microscope and stage to reach 37°C, which improves the maintenance of focus during time-lapse movies.
2. 30–60 minutes before imaging a plate, replace the macrophage medium with Macrophage Imaging Medium. If THP-1 cells are being used for imaging, use THP-1 Imaging Medium for this step. Return cells to the incubator to equilibrate.
3. Just before imaging, overlay the medium with 1–2 mL of mineral oil to prevent evaporation. Place a drop of microscope oil on the bottom of the dish and on the 100X objective.
4. Find a cell(s) with strong expression of pRuby-Lifeact and pEGFP-vinculin. If possible, use neutral density filters when searching for the cells of interest to avoid photobleaching. While it is important to find a cell with strong expression, extremely strong expression may actually decrease resolution and should be avoided.
5. With the filters that will be used during the time lapse, determine the optimal exposure time. In order to minimize photobleaching, choose an exposure time that

is no more than 20–50% longer than needed to obtain a clear image (*see* Note 9). It is important to strike a good balance between image resolution and exposure time so that multiple images may be acquired with minimal photobleaching and toxicity.

6. Acquire a multi-dimensional time-lapse. Take an image of the red and green fluorescence channels, as well as a DIC or phase contrast image every 15–30 seconds for 15–60 minutes. Because of the rapid movement and turnover of podosomes, it is necessary to take images at short intervals. In an active cell, podosome organization can completely change in 1–5 minutes.
7. Additional movies may be made in parallel if your microscope is equipped with an automated stage. Sequential movies may also be made.

3.6 Quantification of Podosome Turnover

1. After acquiring a time-lapse of macrophage podosomes, use a filter to decrease background fluorescence in the image. We use a filter based on the algorithm described by Zamir et al. that works well for reducing diffuse signal around punctate structures (31). Alternatively, most image processing software has the ability to subtract background fluorescence. Applying these filters should increase the resolution of the podosome cores and facilitate their tracking.
2. Scan through the movie to find a podosome that appears and disappears completely during the course of the movie. Make note of the frames at which the podosome is first visible and at which it has completely disappeared. The time at which the podosome initially appears is $t = 0$. Because the rate of formation and dissolution is based on fluorescence intensity, it is important to find a podosome that does not contact other podosomes during its lifetime. If the podosomes are moving actively, no other podosome should move into the path taken by the podosome being analyzed.
3. Create a region that encompasses the area occupied by the podosome of interest during its entire lifetime, but which will not contain other podosomes. Transfer this region to all images in the time-lapse that contain the podosome of interest.
4. Determine the integrated intensity of the region for all relevant points and log this data to a spreadsheet. Record the corresponding times, which are determined by the interval at which the time lapse was taken. Integrated intensity is the sum of the intensity of all the pixels in the region. It should increase and decrease as the podosome assembles and disassembles, respectively. At this point, plotting intensity vs. time would result in a period of exponential assembly and disassembly.

⁹In order to prevent photobleaching of the samples, it is necessary to minimize the exposure times used for acquisition. However, it is prudent to use an exposure time that is longer than the absolute minimum, knowing that some photobleaching will occur. We typically use the following exposure settings: exposure time = 10–100 ms, gain = 1, and pixel binning = 2. In order to decrease exposure time, gain may be increased to 2, and binning may be increased to 3 or 4. However, both of these changes will lead to decreased resolution. Additionally, the use of neutral density filters in the excitation path can substantially decrease the rate of photobleaching. If a confocal microscope is being used, minimize the laser power as much as possible.

5. To make this data linear, generate a semi-logarithmic plot by taking natural logs of the intensity using the following guidelines. To determine the rate of assembly, take the natural log of the product of the intensity (I) at each time point from $t = 0$ (I_0) until the intensity has reached a plateau (I_{Max}) divided by the intensity at $t = 0$.

$$\text{Assembly: } \Delta I = \ln \frac{[I_{0 \rightarrow Max}]}{[I_0]}$$

The change in intensity during disassembly is calculated in a similar manner. Here I_{Max} is the intensity in the frame just prior to the beginning of disassembly and I_{End} is the intensity of the frame in which the podosome is no longer visible.

$$\text{Disassembly: } \Delta I = \ln \frac{[I_{Max}]}{[I_{Max \rightarrow End}]}$$

6. Create a scatter plot for both assembly and disassembly by plotting the change in intensity vs time. Fit a linear trend line to the data and take the slope of this line as the rate constant of assembly or disassembly. As the intensity value has arbitrary units, the calculated rate constant has units of time^{-1} . In order to determine if this model can accurately be applied to the podosome of interest, determine the R^2 value. An $R^2 > 0.7$ is generally acceptable for this analysis.
7. Because podosomes in different parts of the cell may have different rates of assembly, sample podosomes from multiple locations within the cell to determine accurate average rates. Where possible, we determine the assembly/disassembly rates of 10–25 podosomes per cell for 5–10 cells in each experimental condition.

3.7 Production of Gelatin-Coated Cover Slips

The matrix-degrading ability of podosomes is unique in comparison to other types of adhesions. Macrophage podosomes are able to degrade matrix through the secretion of matrix metalloproteinases (32). Podosomes in Src-transformed fibroblasts and osteoclasts have also demonstrated the ability to degrade matrix through the secretion of lysosomal contents (33, 34). While this function has not been directly demonstrated in macrophages, lysosomes colocalize with podosome rosettes in macrophages, and the lysosome-associated protein p61Hck plays a role in podosome formation (12, 35).

Gelatin coated cover slips are a useful tool for studying the degradative ability of podosomes, as they provide both a qualitative result and are amenable to quantification (Figure 4). The following protocol describes a method adapted from Artym et al. for coating cover slips with fluorescent gelatin (36). We have used Oregon Green 488-conjugated gelatin, but gelatin conjugated to other fluorophores may be substituted. Additionally, we demonstrate the techniques for imaging and quantifying podosome-mediated gelatin degradation.

1. Sterilize 24 acid-washed cover slips by dunking in 70% EtOH, and place them on parafilm. Remove residual EtOH by washing each cover slip twice with 1 mL PBS washes (*see* Note 10).
2. Add 150 μ L of 50 μ g/mL poly-L-lysine to each cover slip, allowing the liquid to form a dome over the cover slip. Incubate for 10 minutes at room temperature, aspirate the PLL, and wash once with 1 mL of PBS. For this and all subsequent washes in this protocol, leave $\sim$ 100 μ L of PBS in a dome on the cover slip, and aspirate it just prior to adding the next wash or reagent.
3. Add 150 μ L of 0.5% glutaraldehyde. Incubate for 10 minutes at room temperature. Aspirate the glutaraldehyde and wash each cover slip 5 times with 1 mL of PBS.
4. Line the bottom of a dark box with a sheet of parafilm. Place several 50 μ L drops of the 200 μ g/mL gelatin solution on the parafilm, being careful to avoid bubbles.
5. Aspirate the previous wash from a few cover slips at a time. Lift them from the parafilm using fine tweezers, and invert them on to the gelatin drops. The glutaraldehyde-treated face should be in contact with the gelatin, and there should be no air bubbles. After all of the cover slips have been transferred in this manner, cover the box and incubate at room temperature for 15 minutes.
6. During this incubation, prepare the 5 mg/mL NaBH₄.
7. Prepare a fresh sheet of parafilm. Lift the cover slips off of the gelatin solution and place them gelatin side up on the fresh sheet of parafilm.
8. Place 150 μ L of NaBH₄ on the cover slips, and incubate at room temperature for 10 minutes. NaBH₄ is a reducing agent that quenches any remaining glutaraldehyde. Aspirate the NaBH₄, and transfer the cover slips to a 24 well plate for use or storage. Wash the cover slips twice with 1 mL of PBS.
9. Add 1 mL of PBS to each well to keep the cover slips hydrated until use. The cover slips can be stored at 4°C for 2–4 weeks if they are protected from light.

3.8 Imaging Podosome-Mediated Gelatin Degradation

1. Draw blood and differentiate macrophages as described in section 3.1. On day 6–7 following the blood draw, the macrophages will be ready for use. (If THP-1 cells are being used differentiate them as described in section 3.2.)
2. Lift and count the cells as described in step 7 of section 3.4. Pellet the cells at 500 $\times g$ for 5 minutes. Resuspend the cells in a volume of macrophage medium that will result in a concentration of $\sim 1 \times 10^5$ cells/mL based on the first count. (If THP-1 cells are being used, lift the cells by washing once with PBS and adding 2 mL of accutase. Incubate the dish at room temperature for approximately 5–10 minutes. Cells which remain adherent can be lifted by pipetting. Resuspend the THP-1 cells

¹⁰For all procedures requiring the manipulation of cover slips the choice of forceps is important. We find that very fine-tipped forceps help substantially when lifting cover slips from parafilm or the bottom of 24-well plates.

as described in this step for primary macrophages, but use THP-1 medium. Proceed with step 3.)

3. Perform a final count of the macrophages and dilute them to 6×10^4 cells/mL.
4. Remove a gelatin-coated cover slip from the 24-well plate and place it in a new 24 well plate. Add 3×10^4 macrophages (500 μ L) to the cover slip.
5. Incubate at 37°C with 5% CO₂ for 3–24 hours, depending on the amount of degradation desired. A time course may be taken by fixing cover slips at chosen time intervals.
6. To fix the cover slips, aspirate the medium from each well, wash once with 500 μ L of PBS, and add 500 μ L of Fixative to the wells. Allow the cells to fix for 10 minutes at room temperature.
7. Aspirate the Fixative, and 500 μ L of Quenching Solution for 10 minutes at room temperature.
8. Aspirate the Quenching Solution, and add 500 μ L of Blocking Solution for 30 minutes at room temperature. If you are taking a time course, place the cover slips at 4°C until all of the cover slips are ready for staining.
9. Without aspirating the Blocking Solution, carefully lift cover slips out of their well and place them in a parafilm-lined, dark, plastic box. Add 100 μ L of rhodamine phalloidin diluted 1:1000 in blocking buffer to the top of the cover slips, allowing it to form a dome (*see* Note 11). Incubate for 45 minutes at room temperature.
10. Aspirate the rhodamine phalloidin, and add 100 μ L of DAPI solution. Incubate for 5 minutes at room temperature.
11. Wash 3 times with 1 mL of PBS. Leave ~100 μ L of the PBS on the cover slips for 5–10 minutes between each wash.
12. Spot 7 μ L of mounting medium (MM) on a clean glass slide, being careful not to introduce bubbles. Aspirate the final wash, and invert the cover slip on to the MM. Allow the MM to dry overnight at room temperature in a dark place.
13. Seal the edges of the cover slip with clear nail polish. Allow the nail polish to dry completely before use. After the nail polish has dried, cover slips should be stored at –20°C when not in use.
14. Using a microscope with a 100X objective, take pictures using filters appropriate for rhodamine phalloidin, GFP, DAPI, and DIC/phase contrast. In determining the percent of cell area degraded, the DIC or rhodamine phalloidin image will be used to define the cell area.

¹¹The cover slips are transferred to parafilm at this step to minimize the volume required for staining and minimize the use of expensive reagents. The cover slips may be stained in the plate, but it requires a substantially greater volume to completely coat the cover slip.

3.9 Quantification of Gelatin Degradation

The quantification of podosome-mediated gelatin degradation raises several technical challenges. The areas of degradation are generally irregular in size and shape. Partially degraded areas may retain some fluorescence, creating a gradient of degradation rather than a sharply defined area of degradation. Additionally, even when images are acquired at the same exposure, the fluorescence intensity of the gelatin may vary between cover slips or even regions of the same cover slip. Therefore, we have developed a protocol for defining degraded areas based on the fluorescence intensity of undegraded gelatin within each individual image. The intensity of undegraded gelatin is used as the basis for defining a threshold that encompasses areas of degraded gelatin. We believe that this method reduces the error produced by manually assigning areas of degradation.

1. Open the DIC or rhodamine phalloidin image so that the edge of the plasma membrane can easily be identified.
2. Determine the cell border. Manually trace a region around the plasma membrane, and transfer this region to the corresponding area of the gelatin image.
3. Define the average intensity of undegraded gelatin as a reference. Place a 100×100 pixel region in an area of the gelatin image that contains no cells or areas of degradation. If there is variable intensity across the image, more than one reference region may be required. Determine the average intensity of the reference region (in arbitrary units of intensity). We will define this value as $\text{Int}_{\text{undeg}}$.
4. Determine the value of the lowest intensity represented in the gelatin image. We will define the lowest intensity of the whole image as Int_{lo} .
5. Determine the high threshold value for your image. In order to determine the areas of degradation in the image, it is necessary to calculate the value for the high threshold for the image ($\text{thresh}_{\text{hi}}$). The value for $\text{thresh}_{\text{hi}}$ will be defined by the following equation:

$$\text{thresh}_{\text{hi}} = \text{Int}_{\text{lo}} + ((\text{Int}_{\text{undeg}} - \text{Int}_{\text{lo}}) \cdot x)$$

where x is the “degradation coefficient.” (see Note 12) We find that a degradation coefficient of 0.3–0.6 is a good starting point for our analyses, but it needs optimization based on experimental conditions. (see Note 13)

6. Apply a threshold to the image so that areas with intensities less than $\text{thresh}_{\text{hi}}$ are selected. This threshold should highlight degraded areas in the image.

¹²To determine $\text{thresh}_{\text{hi}}$, we first subtract the lowest intensity represented in the image (Int_{lo}) from the average intensity of undegraded gelatin ($\text{Int}_{\text{undeg}}$) to normalize the differences that will be compared across a series of images. The degradation coefficient, is the fraction of this difference which will be added to Int_{lo} , thereby determining the value of $\text{thresh}_{\text{hi}}$. The values of the degradation coefficient may range from 0–1.

¹³The degradation coefficient used in this calculation must be determined by the user. When we quantify gelatin degradation in this manner, we try several values on a subset of our images to determine which value of the degradation coefficient best represents the actual degradation. After a value is chosen for the degradation coefficient, it remains constant throughout the experiment and replicates of the experiment. We suggest testing values in the range of 0.3–0.6 because this is the range that has worked for us in several conditions.

7. Determine the area of the cell that was traced in steps 2 and 3. Determine the area of the thresholded region that falls within the borders of the cell. The percent of cell area degraded is as follows:

$$\text{Percent Degraded} = \frac{\text{Area Thresholded}}{\text{Total Cell Area}}$$

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Figure 1. Comparison of podosomes, invadopodia, and focal adhesions

(a) Primary human macrophages differentiated with MCSF for 7 days and (b) THP-1 cells differentiated for 48 hours after PMA treatment were stained with rhodamine phalloidin and an anti-vinculin antibody. Note the prominent ring structures in the vinculin images that are characteristic of podosomes. (c) MTLn3 rat mammary carcinoma cells stained with rhodamine phalloidin and an anti-vinculin antibody. Note the localization of vinculin to focal adhesions, which are formed at the termini of actin stress fibers (arrows), and the absence of vinculin rings in areas corresponding to the punctate invadopodia (arrowheads). Scale bar = 10 μ m for all panels.

Figure 2. Schematic of Podosome Structure

Pictured on the top right is a cell forming a cluster of podosomes as viewed from above. The gross podosome structure is depicted as black dots and grey rings. Below is a magnified cross section of an individual podosome. The colors of the proteins in the cross-section correspond to their location in the podosome core (black) or ring (grey). Matrix metalloproteinase 9 (MMP-9) is secreted from podosome cores and is capable of degrading the underlying extracellular matrix (ECM). Degraded matrix is depicted as dashed lines.

Figure 3. Live fluorescent imaging of podosomes in primary human macrophages

Primary human macrophages were differentiated for 7 days with MCSF. On day 7, the macrophages were nucleofected using 0.5 μg of pRuby-Lifeact and 0.5 μg of pEGFP-vinculin and plated on fibrinogen-coated glass bottom dishes. Time-lapse images were acquired using the following settings: binning = 3×3 , gain = 2, Lifeact exposure = 15 ms, and vinculin exposure = 15 ms. Images were taken at 1 minute intervals for 20 minutes. Lifeact and vinculin images are shown at 2 minute intervals. Arrows indicate assembling or disassembling podosomes. Scale bar = 10 μm.

Figure 4. Gelatin degradation by primary macrophages

Cover slips were coated with 200 $\mu\text{g/mL}$ Oregon Green-488 gelatin. 3×10^4 primary human macrophages, which had been differentiated for 7 days with MCSF, were added to the cover slips. Podosomes were stained with rhodamine phalloidin. To determine the percentage of cell area degraded, a degradation coefficient of 0.3 was used for each image. The white shading in the right-most column indicates the area that was thresholded as degradation. The percentage of cell area degraded is indicated. Images were selected to demonstrate various degrees of degradation. Scale bar = 10 μm .

Table 1**Components Common to Podosomes and Invadopodia**

Component	Function	Reference
Actin	Cytoskeletal	A
Arp 2/3	Actin-nucleation	B, C
WASP/N-WASP	Actin-binding	D, E
Cortactin	Actin-binding	F, G
Src kinases	Signaling	H, I, J
Matrix Metalloproteases	ECM Degradation	K, L, M

^A Tarone, G., D. Cirillo, F.G. Giancotti, P.M. Comoglio, and P.C. Marchisio. 1985. Rous sarcoma virus-transformed fibroblasts adhere primarily at discrete protrusions of the ventral membrane called podosomes. *Exp Cell Res.* 159:141–57.

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Table 2**Components Common to Podosomes and Focal Adhesions**

Component	Function	Reference
Talin	Cytoskeletal	<i>A, B</i>
Paxillin	Cytoskeletal	<i>C, D</i>
Zyxin	Cytoskeletal	<i>E, F</i>
Integrins	Cytoskeletal/Signaling	Reviewed in <i>G</i>
Src kinases	Signaling	<i>H, I, J</i>
FAK/Pyk2	Cytoskeletal/Signaling	<i>K, L</i>
Vinculin	Actin-binding	<i>M, N</i>
Tensin	Actin-binding	<i>O, P</i>

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