Video Article

Using Cell-substrate Impedance and Live Cell Imaging to Measure Real-time Changes in Cellular Adhesion and De-adhesion Induced by Matrix Modification

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Abstract

Cell-matrix adhesion plays a key role in controlling cell morphology and signaling. Stimuli that disrupt cell-matrix adhesion (*e.g.*, myeloperoxidase and other matrix-modifying oxidants/enzymes released during inflammation) are implicated in triggering pathological changes in cellular function, phenotype and viability in a number of diseases. Here, we describe how cell-substrate impedance and live cell imaging approaches can be readily employed to accurately quantify real-time changes in cell adhesion and de-adhesion induced by matrix modification (using endothelial cells and myeloperoxidase as a pathophysiological matrix-modifying stimulus) with high temporal resolution and in a non-invasive manner. The xCELLigence cell-substrate impedance system continuously quantifies the area of cell-matrix adhesion by measuring the electrical impedance at the cell-substrate interface in cells grown on gold microelectrode arrays. Image analysis of time-lapse differential interference contrast movies quantifies changes in the projected area of individual cells over time, representing changes in the area of cell-matrix contact. Both techniques accurately quantify rapid changes to cellular adhesion and de-adhesion processes. Cell-substrate impedance on microelectrode biosensor arrays provides a platform for robust, high-throughput measurements. Live cell imaging analyses provide additional detail regarding the nature and dynamics of the morphological changes quantified by cell-substrate impedance measurements. These complementary approaches provide valuable new insights into how myeloperoxidase-catalyzed oxidative modification of subcellular extracellular matrix components triggers rapid changes in cell adhesion, morphology and signaling in endothelial cells. These approaches are also applicable for studying cellular adhesion dynamics in response to other matrix-modifying stimuli and in related adherent cells (*e.g.,* epithelial cells).

Video Link

The video component of this article can be found at https://www.jove.com/video/52423/

Introduction

Stable adhesive contacts between cells and their surrounding extracellular matrix are required for maintenance of tissue homeostasis. For example, endothelial cell adhesion to the subendothelial matrix in blood vessels plays a critical role in maintaining the integrity of the endothelial layer and its homeostatic function as a regulatory, semi-permeable vascular barrier[1]. The actin cytoskeleton is mechanically coupled to adhesive matrix molecules at sites of cell-matrix adhesion and adhesive contacts at the cell-matrix interface play an important role in determining the position of the cell membrane by resisting centrally-directed actomyosin tensile forces. Extracellular stimuli that alter cell-matrix adhesion necessarily alter the balance of forces at the cell-matrix interface, an event that is rapidly 'sensed' by mechano-sensitive signaling proteins, resulting in the transduction of "outside-in signaling". This cross-talk between cells and their surrounding extracellular matrix plays a key role in controlling cell shape, motility, function, proliferation and survival[2].

Diverse patho-physiological processes (embryonic development, inflammation, wound repair and cancer metastasis) are characterized by dynamic remodeling of adhesive matrix substrates by matrix-degrading oxidants and/or enzymes[3,4]. For example, adhesive subendothelial matrix proteins in blood vessels (*e.g.*, fibronectin) are implicated as major targets for modification or degradation in human inflammatory diseases due to the localized production of reactive oxidants (*e.g.*, hypochlorous acid, HOCl) by the leukocyte-derived enzyme myeloperoxidase (MPO), which accumulates within the subendothelium during inflammatory vascular disease (**Figure 1**)[5-9]. Changes in cell-matrix adhesion induced by MPO-derived oxidants and other matrix-modifying stimuli are likely to play important roles in altering vascular homeostasis during a variety of pathological processes; *e.g.*, by altering endothelial cell signaling, morphology and viability, which in turn perturbs endothelial function and barrier integrity. However, the morphological and cell signaling responses of adherent cells to extracellular matrix modifications are only beginning to be understood.

To understand how matrix modifications drive changes in cell adhesion dynamics and adhesion-dependent cell signaling pathways, techniques are required that accurately quantify changes in cell-matrix adhesion in real time, with high temporal resolution. Here, we describe

complementary cell-substrate impedance and live cell imaging techniques that fulfill these criteria and provide a platform to quantify cell adhesion and de-adhesion processes in a non-invasive manner.

We show how these cell-substrate impedance and live cell imaging approaches can be readily employed to (i) monitor the dynamics of cell attachment and spreading (*i.e.*, *de novo* cell adhesion) onto native and modified matrix substrates and (ii) to measure the dynamics of cell-matrix detachment (*i.e.*, de-adhesion) by adherent cells exposed to matrix-modifying stimuli. The xCELLigence cell-substrate impedance biosensor system provides a continuous measurement of the area of cell-matrix contact by quantifying electrical impedance at the surface of 96-well gold microelectrode arrays and expresses these electrical impedance measurements as 'cell index', a dimensionless value that is largely proportional to the area of cell-substrate contact[10] (**Figure 2**), whilst also being sensitive to changes in the average distance between the (insulating) cell membrane and the electrode surface[11]. A further increase in cell index values is also achieved upon formation of tight cell-cell contacts that restrict paracellular current flows,[11] conditions that do not prevail within the experiments described in this study. Measurement of the projected area of individual cells over time by image analysis of time-lapse differential interference contrast (DIC) movies provides a complementary measure of changes in the area of cell-substrate contact and provides additional information regarding the precise nature and dynamics of the morphological changes quantified by the cell-substrate impedance approach.

Specifically, we describe the application of these approaches to monitor how MPO-mediated oxidation of adhesive subendothelial matrix proteins (*e.g.*, fibronectin) (i) reduces the *de novo* adhesion of suspended endothelial cells onto purified fibronectin and (ii) triggers cell-matrix de-adhesion in endothelial cells with established adhesion on fibronectin. By performing parallel cell signaling analyses over time using relevant biochemical assays (*e.g.*, Western blotting), the temporal and causal relationships between adhesion/de-adhesion processes and associated changes in adhesion-dependent cell signaling events can be determined.

These approaches were recently used to demonstrate that extracellular matrix oxidation catalyzed by subendothelial deposits of MPO triggers a rapid loss in cell-matrix adhesion of endothelial cells that is driven by pre-existing actomyosin contractile forces[9]. Importantly, by enabling the temporal relationship between changes in both cell adhesion and adhesion-dependent cell signaling to be determined, these approaches identified that MPO-induced matrix modification and cellular de-adhesion triggers changes in important adhesion-dependent cell signaling pathways including Src kinase-dependent paxillin phosphorylation and myosin light chain II phosphorylation (**Figure 1**)[9]. This mode of redox-dependent signaling, involving the activation of intracellular signaling events by extracellular oxidative reactions that disrupt cell-matrix adhesion, represents a novel mode of cell signaling termed "outside-in redox signaling" (**Figure 1**)[9].

In general, these complementary cell-substrate impedance biosensor and live cell imaging approaches should be valuable in revealing how different matrix-modifying stimuli or agents drive changes in cell adhesion dynamics, morphology and signaling within different adherent cell-types subject to a wide variety of experimental settings.

The following protocol describes how to quantify the impact of MPO-mediated matrix oxidation on *de novo* endothelial cell adhesion (**Experiment 1**) and endothelial cell de-adhesion (**Experiment 2**) processes. MPO binds avidly to fibronectin and other adhesive subendothelial extracellular matrix proteins and uses hydrogen peroxide (H_2O_2) to convert chloride ions (Cl^-) to the highly reactive chlorinating oxidant hypochlorous acid (HOCl), which reacts locally with these matrix proteins and disrupts their cell adhesive properties (**Figure 1**)[8,9,12].

Protocol

1. General Endothelial Cell Culture

1. Culture bovine aortic endothelial cells (passages 4–9) on gelatin-coated tissue culture flasks (coat tissue culture surface with 0.1% w/v gelatin in PBS at RT for 15 min) in EGM-2 media (with the EGM-2 bullet kit containing 5% fetal bovine serum, growth factors and all supplements provided by the manufacturer, except for hydrocortisone).
2. When cells are near-confluent (ca. 3 days post seeding after a 1:4 split), harvest cells by treatment with 0.05% w/v trypsin / 0.02% w/v EDTA in PBS at 37 °C. After the majority of cells have detached, add complete EGM-2 media to quench the trypsin and then centrifuge (100 x g, 5 min).
3. Prepare cells for studies on the *de novo* adhesion of endothelial cells to fibronectin (Experiment 1: Section 2) and the subsequent de-adhesion of endothelial cells with established adhesion on this substrate (Experiment 2: Section 3).
    1. Wash harvested cells once with serum-free Medium 199 containing 1% w/v bovine serum albumin (BSA) and re-centrifuge (100 x g, 5 min).
    2. Re-suspend cells in serum-free Medium 199 containing 1% w/v BSA at 2.5 x 10^5 cells/ml (cell-substrate impedance measurements) or 5 x 10^5 cells/ml (live cell imaging analyses) and maintain at 37 °C prior to use.
    NOTE: The adhesion responses of cells are highly sensitive to temperature differences (*e.g.,* due to convection effects) so all equipment and solutions used to handle and treat cells during the following protocols should be kept at a constant temperature of 37 °C.

2. Experiment 1: Quantifying *De Novo* Endothelial Cell Adhesion on Native and MPO-oxidized Fibronectin (Cell-substrate Impedance)

NOTE: Experiment 1 examines the degree to which MPO-mediated oxidation of fibronectin impairs its ability to support *de novo* adhesion of suspended endothelial cells.

1. Coat fibronectin onto 96-well gold cell-substrate impedance microelectrode arrays. Add 80 µl/well of purified bovine fibronectin at 5 µg/ml in PBS, incubate for 2 hr at 37 °C and remove the solution.

2. Incubate fibronectin-coated surfaces with MPO to allow the binding of MPO to the surface bound fibronectin. Add 80 µl/well of purified human neutrophil MPO at 20 nM in Hank's balanced salt solution (HBSS) and incubate for 0.5 hr at 37 °C.
3. Wash surfaces twice with HBSS to remove any unbound MPO.
4. Add H_2O_2 (0-10 µM final concentration) to wells of the microelectrode array plate containing 80 µl/well HBSS to initiate MPO-catalyzed, HOCl-dependent fibronectin oxidation and incubate for another 0.5 hr at 37 °C.
5. To examine the effect of relevant inhibitors or modulators of MPO-catalyzed reactions (*e.g.*, alternative MPO enzyme substrates, enzyme inhibitors or antioxidants; see[9] for details), add these to the HBSS immediately prior to H_2O_2 addition.
6. After 0.5 hr, treat surfaces with methionine to quench residual surface-bound oxidizing species; *i.e.*, reactive protein-bound chloramines, which may exert confounding cellular activities. Add 10 mM methionine in 80 µl HBSS per well and incubate for 10 min at 37 °C.
7. Block surfaces with BSA. Add 80 µl/well of BSA at 0.2% w/v in PBS, incubate for 2 hr at 37 °C and remove the solution.
   NOTE: Residual uncoated surface regions will support cell adhesion and blocking these with a non-adhesive protein (*i.e.*, BSA) ensures that cellular adhesion responses strictly depend on the purified cell-adhesive matrix employed, in this case fibronectin.
8. Wash surfaces twice with HBSS.
   NOTE: None of the preceding surface treatments appreciably affects cell-substrate readings.
9. Seed suspended endothelial cells (add 200 µl/well at ca. 2.5 x 10^5 cells/ml, prepared in serum-free Medium 199 containing 1% BSA; see 1.3) onto the native or MPO-oxidized fibronectin coated surfaces.
10. Immediately after seeding cells (*i.e.*, prior to any cell attachment and spreading), mount the microelectrode array plate onto the incubator port (housed in a 37 °C incubator in the presence of 5% CO_2).
    1. Using the instrument software immediately take a 'blank' reading to normalize subsequent cell-substrate impedance ('cell index') values to the initial background values obtained in the absence of cell-adhesion.
    2. Initiate acquisition of continuous cell index data (minimum of one cell index reading/min).

11. Incubate cells at 37 °C and 5% CO_2 for 2 hr, a time period during which maximal cell attachment and spreading is achieved; this is reflected by a plateauing of the cell index values (see **Figure 3A**).
    NOTE: The preceding experiment (Experiment 1) examines how initial MPO-mediated oxidation of fibronectin limits the ability of endothelial cells to establish cell adhesion on this substrate. The following experiment (Experiment 2) examines how MPO-mediated fibronectin oxidation promotes decreases in cell-matrix adhesion (*i.e.*, de-adhesion) in endothelial cells with established adhesion onto this substrate. The treatments in these two experiments are essentially identical, except for the timing of MPO-mediated fibronectin oxidation (*i.e.*, before cell adhesion – Experiment 1; after cell adhesion – Experiment 2).

3. Experiment 2: Quantifying Endothelial Cell De-adhesion from Fibronectin in Response to MPO-mediated Fibronectin Oxidation (Cell-substrate Impedance and Live Cell Imaging)

1. Coat fibronectin onto 96-well gold microelectrode arrays for cell-substrate impedance measurements (add 80 µl/well of fibronectin at 5 µg/ml in PBS and incubate for 2 hr at 37 °C) or 35 mm glass-bottomed cell culture dishes for live cell imaging analyses (add 2 ml/dish of fibronectin at 5 µg/ml in PBS and incubate for 2 hr at 37 °C).
2. Block surfaces with BSA. Add BSA at 0.2% w/v in PBS at the volumes indicated in 3.1 and incubate for 2 hr at 37 °C.
3. Incubate surfaces with MPO to allow the binding of MPO to fibronectin. Add 20 nM purified human MPO in HBSS at the volumes indicated in 3.1 and incubate for 0.5 hr at 37 °C.
4. Wash surfaces twice with HBSS to remove any unbound MPO.
5. Seed suspended endothelial cells (prepared in serum-free Medium 199 containing 1% w/v BSA; see 1.3) onto the native or MPO-bearing fibronectin coated surfaces.
   1. Add 200 µl/well at 2.5 x 10^5 cells/ml to 96 well cell-substrate impedance microelectrode arrays.
   2. Add 2 ml/dish at 5 x 10^5 cells/ml to 35 mm glass bottomed cell culture dishes.

6. Immediately after seeding cells (*i.e.*, prior to any cell attachment and spreading):
   1. Mount 96 well microelectrode array plates onto the cell-substrate impedance incubator port (housed in a 37 °C incubator in the presence of 5% CO_2) and take 'blank' readings to initiate continuous acquisition of cell index data (cf. Section 2.10).
   2. Transfer 35 mm glass bottomed cell culture dishes to a 37 °C incubator in the presence of 5% CO_2.

7. Incubate cells at 37 °C for 2 hr to allow maximal cell attachment and spreading (cf. Section 2.11).
8. Briefly remove the 96 well microelectrode array plate (pause cell index readings at this point) or 35 mm glass bottomed cell culture dish from the 37 °C incubator, remove cell supernatant and add warmed (37 °C) HBSS (volumes as per step 3.1).
   NOTE: HBSS is employed when studying MPO-catalyzed oxidative reactions instead of complete culture media as the latter contains oxidizable species that interfere with the oxidation reactions.
9. To examine the effect of inhibitors/modulators of MPO-catalyzed reactions (alternative enzyme substrates, enzyme inhibitors or antioxidants) or cell signaling inhibitors (*e.g.*, 40 µM blebbistatin to inhibit actomyosin contractility), add these now.
10. Immediately place the microelectrode array plate back into the 37 °C incubator port (re-commence cell index readings at this point) or the 35 mm glass bottomed cell culture dish back into the 37 °C incubator, and allow the cells to equilibrate in the HBSS for 0.5 hr.
    NOTE: After 0.5 hr equilibration in HBSS, cell index values stabilize at slightly lower values; when pre-incubating cells with enzyme substrates, enzyme and cell signaling inhibitors or antioxidants, first ensure that these do not significantly affect the values obtained after equilibration.
11. Initiate MPO-catalyzed, HOCl-dependent fibronectin oxidation and de-adhesion.
    1. For cell impedance studies using 96 well microelectrode array plates:
       1. Remove the microelectrode array plate from the incubator and pause cell index measurements.
       2. Add H_2O_2 (final concentration of 0-10 µM) to the HBSS and gently mix by repeated pipetting.
       3. Immediately, re-mount the microelectrode array back onto the 37 °C incubator port and re-commence the acquisition of cell index readings.

2. For live cell imaging studies using 35 mm glass bottomed cell culture dish:
    1. Remove the culture dish from the incubator and mount onto a 37 °C heated stage of an inverted confocal microscope equipped with a 63X water objective lens with suitable DIC optics for recording live cell movies.
    2. Focus on cells and optimize DIC optics (Köhler illumination, bias retardation and camera offset/gain; for details, see[13]).
    3. Initiate DIC movie and record baseline readings of untreated cells for 1 min.
    4. Add H_2O_2 (final concentration of 0-10 µM) and gently mix by repeated pipetting. Re-focus the microscope (if necessary) and continue recording DIC movies of the treated cells for the required time period.

4. Data Analysis and Presentation

1. Cell-substrate impedance data
    1. Export raw data (cell index versus time) into a spreadsheet.
    2. For cell de-adhesion studies (Experiment 2: Section 3), normalize data by setting values recorded immediately prior to the initiation of MPO-mediated fibronectin oxidation at a value of 1 (*i.e.*, immediately before the addition of H_2O_2 in 3.11.1.1).
    NOTE: This ensures that relative changes in cell index values elicited by MPO-mediated fibronectin oxidation are not masked by small initial differences in absolute cell index values between wells.
        1. Present data as plots of normalized cell index (y-axis) versus time (x-axis).

2. Live cell imaging data (cell de-adhesion studies in Experiment 2: Section 3)
    1. Open DIC live cell imaging movies recorded immediately prior to and after the initiation of MPO-mediated fibronectin oxidation in a standard image analysis program (*e.g.,* ImageJ software).
    2. In at least two separate DIC movies randomly select multiple cells and measure their projected area in sequential frames (*e.g.,* at 1 min intervals) by manually tracing their membrane edge and quantifying the number of enclosed pixels.
    3. Export raw data (projected cell area versus time) to an Excel spreadsheet and normalize cell area data by setting values recorded immediately prior to the initiation of MPO-mediated fibronectin oxidation at a value of 1 (*i.e.*, immediately before the addition of H_2O_2 in 3.11.2.3).
    4. Present data as a plot of normalized cell area (y-axis) versus time (x-axis).

Representative Results

Real-time quantification of endothelial cell de-adhesion from fibronectin in response to MPO-mediated fibronectin oxidation (Experiment 2). The seeding of endothelial cell suspensions onto native (MPO free) fibronectin or MPO-bearing fibronectin results in maximal cell attachment and spreading within 2 hr, as judged by a plateauing of cell index values in the cell substrate impedance measurements (**Figure 3A**). This initial phase of cell attachment and spreading is markedly reduced when MPO-mediated fibronectin oxidation is initiated prior to cell seeding in experiments performed according to the protocol detailed in Experiment 1 (data not shown; For details see[9]). Once maximal cell adhesion is established on native or MPO-bearing fibronectin, targeted fibronectin oxidation mediated by MPO-catalyzed HOCl (initiated by the addition of MPO's co-substrate H_2O_2) causes a rapid decrease in the surface area of cell-matrix contact measured by cell-substrate impedance (**Figure 3A,B**) and by live cell imaging (**Figure 3C**; for a representative DIC movie, see **Movie 1**; cell index or cell area losses after H_2O_2 addition are minimal in the absence of MPO[9]). Whilst cell index and cell area changes during MPO-induced de-adhesion were very similar, the comparatively slower decreases in cell index values may reflect the insulating effects of cell membrane materials present in 'cell-free' regions at the cell periphery during de-adhesion, which are not quantified in the projected cell area measurements. The rapid cellular de-adhesion apparent in endothelial cells bound to MPO-bearing fibronectin in response to H_2O_2 treatment is absent in cells treated with H_2O_2 alone (*i.e.*, cells that do not contain MPO) (**Figure 3A**) and is blocked by MPO enzyme inhibitors or HOCl scavengers (data not shown; see[9] for details), identifying that this process is dependent on the MPO-catalyzed production of HOCl (cf. **Figure 1**). Inhibition of myosin II motor function with blebbistatin inhibits the rate of endothelial cell de-adhesion measured by cell-substrate impedance (**Figure 3B**) and by live cell imaging (**Figure 3C**, **Movie 2**), identifying that cellular de-adhesion and contraction in response to MPO-catalyzed subcellular matrix oxidation is driven by actomyosin tensile forces[9].

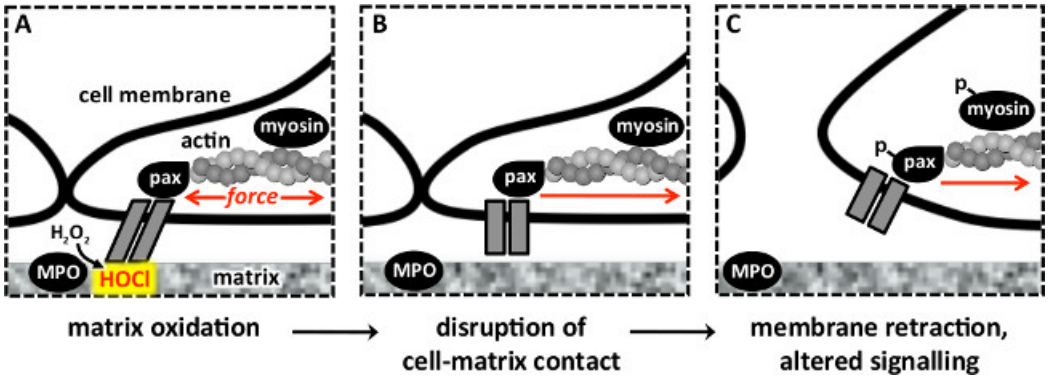

Figure 1. MPO-mediated subcellular matrix oxidation triggers cell-matrix de-adhesion and cell signaling. MPO triggers rapid de-adhesion responses and changes in adhesion-dependent signaling by mediating targeted oxidation of adhesive subendothelial matrix proteins, involving the following events[9]: (**A**) MPO binds avidly to the subendothelial matrix and uses H_2O_2 to generate the highly reactive oxidant HOCl that reacts locally with matrix proteins (*e.g.*, fibronectin) and disrupts their cell adhesive properties. (**B**) This damage disrupts adhesive contacts at the cell-matrix interface, leading to (**C**) membrane retraction driven by unopposed tension in the actin cytoskeleton and the alteration of adhesion-dependent cell signaling pathways (Reproduced with permission from Rees *et al.*[9]). Please click here to view a larger version of this figure.

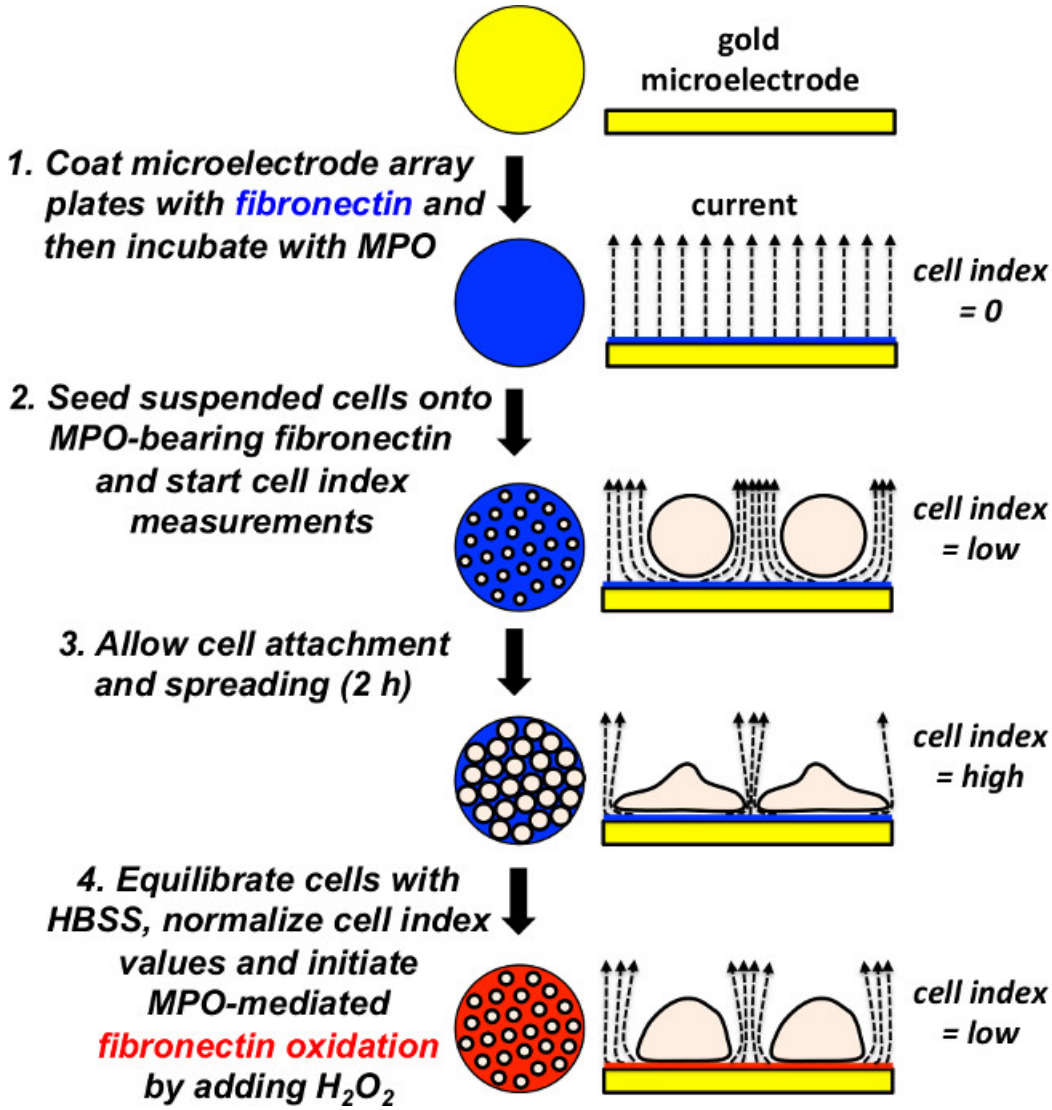

Figure 2. Work-flow and principle of cell-substrate impedance analyses to quantify endothelial cell de-adhesion from fibronectin in response to MPO-mediated fibronectin oxidation (Experiment 2). Cell index values measured by the cell-substrate microarray impedance biosensor system reflect the ability of the cell membrane to limit field-induced ion currents at the electrode surface and are proportional to the area of cell–substrate contact[10]. Please click here to view a larger version of this figure.

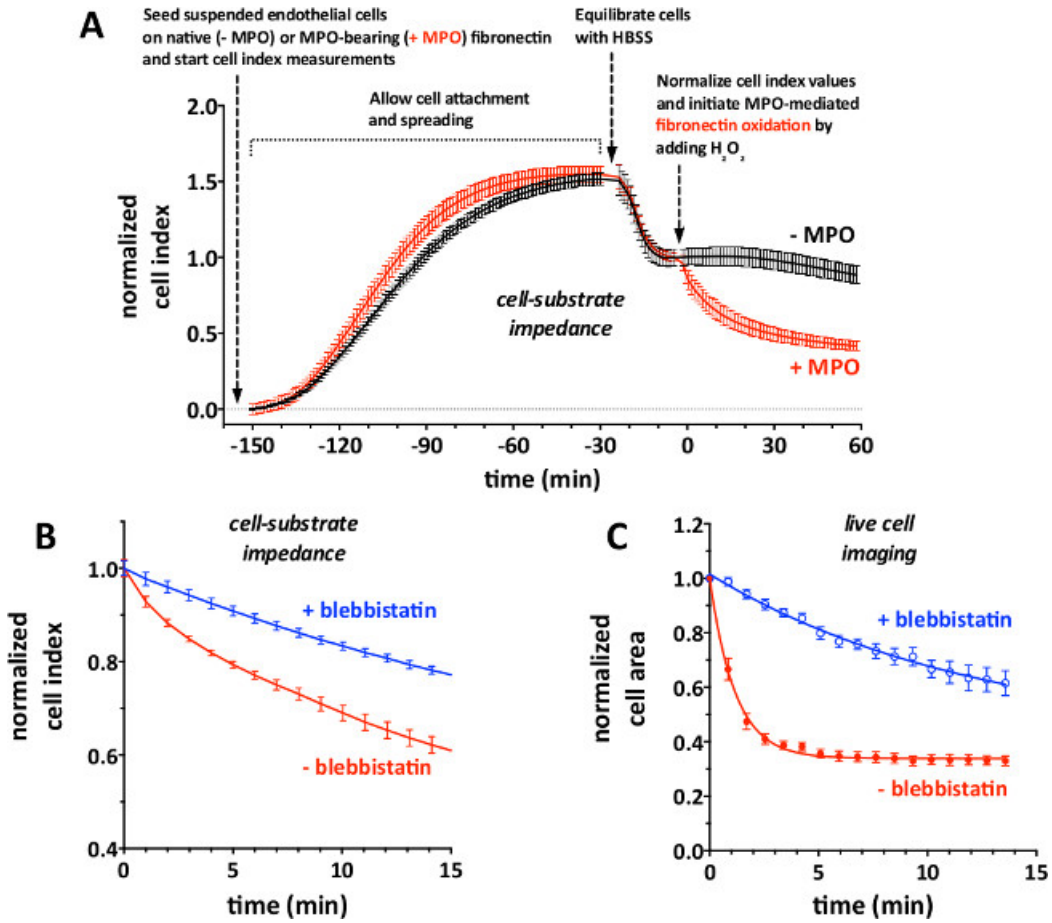

Figure 3. Real-time quantification of endothelial cell de-adhesion from fibronectin in response to MPO-mediated fibronectin oxidation (Experiment 2). Endothelial cell suspensions (in serum-free Medium 199 containing 1% BSA) were seeded onto native or MPO-bearing fibronectin (fibronectin coated at 5 g/ml, then incubated in the absence or presence of 20 nM MPO) in 96 well microelectrode arrays (cell-substrate impedance measurements) or 35 mm glass-bottom cell culture dishes (live cell imaging analyses). Cells were then incubated at 37°C for 2 hr to allow maximal cell attachment and spreading before equilibrating cells with HBSS and initiating MPO-mediated fibronectin oxidation by adding H_2O_2 (10 μM) (time of H_2O_2 addition set at t = 0 min). (**A**) Full time-course of cell-substrate impedance measurements before and after treatment with H_2O_2 in the presence (+MPO) and absence (-MPO) of MPO. (**B**) Cell-substrate impedance measurements and (**C**) cell area measurements by live cell imaging analyses, after treatment of MPO-containing cells with H_2O_2 in the presence (+ blebbistatin) and absence (- blebbistatin) of the myosin II inhibitor blebbistatin (40 μM). Cell index and cell area values are normalized to values immediately prior to the time of H_2O_2 addition, which were given a value of 1. Cell index data represent the mean SEM, n = 6 replicate measurements from a representative experiment. Cell area values represent the mean SEM, n = 10 cells (2 replicate movies, 5 randomly selected cells per movie: see **Movies 1** and **2**). (**Figure 3B**, **Figure 3C**, **Movie 1** and **Movie 2** are reproduced with permission from Rees *et al.*[9]). Please click here to view a larger version of this figure.

Movie 1. Time lapse DIC microscopy movie of endothelial cells adhered on MPO-bearing fibronectin over 0 – 15 min following exposure to H_2O_2 (10 μM); 1 sec = 3 min.

Movie 2. Time lapse DIC microscopy movie of endothelial cells adhered on MPO-bearing fibronectin over 0 – 15 min following exposure to H_2O_2 (10 μM) in the presence of blebbistatin (40 μM); 1 sec = 3 min.

Discussion

Cell-matrix adhesion and de-adhesion processes can be quantified accurately in real time with high temporal resolution using cell-substrate impedance and live cell imaging analyses. These real-time approaches provide a major advantage over end-point analyses of cell adhesion, which provide poor temporal resolution. By accurately quantifying rapid de-adhesion responses with high temporal resolution, these analyses can provide critical insights into how morphological responses to matrix modifications are regulated and how they impact on adhesion-dependent cell signaling processes, measured in parallel using relevant biochemical assays (*e.g.*, Western blotting).

In recent studies, these approaches were used to reveal a novel role for MPO-mediated oxidation of the subendothelial matrix in triggering the de-adhesion of endothelial cells and showed: (i) that the rate of de-adhesion was critically dependent on pre-existing actomyosin contractility (cf. **Figures 3B and 3C**) and (ii) that the loss of cell-matrix adhesion drove changes in important adhesion-dependent cell signaling pathways including Src-dependent paxillin phosphorylation and myosin light chain II phosphorylation[9] (see **Figure 1**). These data have important implications for understanding endothelial function and barrier integrity during inflammatory responses, where the subendothelial extracellular

matrix is implicated as a key target for oxidants produced by subendothelial deposits of matrix-bound MPO or other sources of reactive oxidants [3,5-9,14].

The use of a microarray-based, cell-substrate impedance biosensor system provides a platform for robust, high-throughput cell adhesion measurements. Each well of the 96 well cell-substrate impedance microelectrode arrays has the potential to simultaneously analyze 32 different experimental conditions (*i.e.*, 3 wells per condition). However, when comparing small changes in cell adhesion, at least 4 or more wells should be used per experimental condition. During cell seeding and subsequent cell treatments, care should be taken to keep all solutions at 37 °C and minimize the time taken to treat cells outside the 37 °C incubator environment (this avoids potential temperature differences across the microelectrode array that may affect adhesion responses). Notably, the electrical current at the cell-substrate interface is sensitive to the ionic strength of the cell supernatant[10]: consequently, cells should be allowed to equilibrate after the addition of new buffers/media to obtain a steady cell index response before monitoring the effect of the stimulus of interest (see 3.10 and **Figure 3A**). Decreases in cell index may not only reflect a decrease in the average area of cell-matrix contact but may also reflect a decrease in the number of attached cells. To discriminate between these possibilities, changes in the number of attached cells on microelectrode arrays can be quantified immediately after cell-substrate impedance measurements by Crystal violet staining this approach has been used to confirm that MPO-mediated fibronectin oxidation triggers a rapid decrease in the average area of cell-matrix contact, but does not promote cell detachment (for details, see[9]).

A limitation of the cell-substrate impedance technique is that it provides an average measure of adhesion changes over all cells present on the microelectrode surface and it does not give information about the precise nature of adhesion or morphological changes at the level of individual cells. To address this limitation, live cell DIC microscopy and image analysis provides a complementary measure of adhesion changes and reveals morphological changes of individual cells. Thus, decreases in cell index in response to MPO-mediated fibronectin oxidation measured by cell-substrate impedance (**Figure 3A**) correlate well with the decreases in the projected area of cells measured by live cell image analysis of DIC movies (**Figure 3B**). Importantly, DIC movies reveal that, in individual cells, MPO-induced de-adhesion involves the rapid retraction of the peripheral cell membrane from the substratum and adjacent cells and ultimately results in cells assuming a compact 'rounded-up' morphology, but without resulting in cell detachment (**Movie 1**). As with cell-substrate impedance measurements, to ensure reproducible cellular responses, care should be taken to maintain solutions at 37 °C prior to use on cells and a heated microscope stage (or equivalent temperature control device) must be employed during the live cell imaging recordings.

The protocols described here can be modified readily by substituting fibronectin with other purified matrix substrates (*e.g.*, collagen or laminin) or studying a more complex matrix environment produced overtime by cultured adherent cells (for details, see[9]). The protocols can also be altered by substituting MPO-mediated matrix oxidation with other soluble stimuli that disrupt cell-matrix adhesion including other physiological matrix-modifying oxidants (*e.g.*, peroxynitrite, nitrogen dioxide radical[5-8]), matrix-degrading proteases[15] or antagonists of adhesive ligands present on the cell surface or in the matrix (*e.g.*, anti-integrin antibodies or RGD peptides). Live cell imaging analyses can also be used to quantify cell-matrix de-adhesion in response to the electrochemical desorption of cell-matrix contacts[16]. Finally, the described protocols are also readily applicable to studying cellular adhesion dynamics in adherent cells other than endothelial cells (*e.g.*, epithelial cells).

Disclosures

The authors have no disclosures to make.

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References

1. Wu, M. H. Endothelial focal adhesions and barrier function. *J Physiol.* **569**, 359-366 (2005).
2. Geiger, B., Yamada, K. M. Molecular architecture and function of matrix adhesions. *Cold Spring Harb Perspect Biol.* **3**, (2011).
3. Rees, M. D., Kennett, E. C., Whitelock, J. M., Davies, M. J. Oxidative damage to extracellular matrix and its role in human pathologies. *Free Radic Biol Med.* **44**, 1973-2001 (2008).
4. Cox, T. R., Erler, J. T. Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. *Dis Model Mech.* **4**, 165-178 (2011).
5. Baldus, S., *et al.* Endothelial transcytosis of myeloperoxidase confers specificity to vascular ECM proteins as targets of tyrosine nitration. *J Clin Invest.* **108**, 1759-1770 (2001).
6. Baldus, S., *et al.* Spatial mapping of pulmonary and vascular nitrotyrosine reveals the pivotal role of myeloperoxidase as a catalyst for tyrosine nitration in inflammatory diseases. *Free Radic Biol Med.* **33**, 1010-1019 (2002).
7. Thomas, S. R., Witting, P. K., Drummond, G. R. Redox control of endothelial function and dysfunction: molecular mechanisms and therapeutic opportunities. *Antioxid Redox Signal.* **10**, 1713-1765 (2008).
8. Rees, M. D., *et al.* Myeloperoxidase-derived oxidants selectively disrupt the protein core of the heparan sulfate proteoglycan perlecan. *Matrix Biol.* **29**, 63-73 (2010).
9. Rees, M. D., *et al.* Targeted subendothelial matrix oxidation by myeloperoxidase triggers myosin II-dependent de-adhesion and alters signaling in endothelial cells. *Free Radic Biol Med.* **53**, 2344-2356 (2012).
10. Solly, K., Wang, X., Xu, X., Strulovici, B., Zheng, W. Application of real-time cell electronic sensing (RT-CES) technology to cell-based assays. *Assay Drug Dev Technol.* **2**, 363-372 (2004).
11. Giaever, I., Keese, C. R. Micromotion of mammalian cells measured electrically. *Proc Natl Acad Sci U S A.* **88**, 7896-7900 (1991).
12. Vissers, M. C., Thomas, C. Hypochlorous acid disrupts the adhesive properties of subendothelial matrix. *Free Radic Biol Med.* **23**, 401-411 (1997).

13. Ziv, N. E., Schiller, J. Differential Interference Contrast (DIC) Imaging of Living Cells. *CSH Protoc.* **2007**, (2007).
14. Kennett, E. C., *et al.* Peroxynitrite modifies the structure and function of the extracellular matrix proteoglycan perlecan by reaction with both the protein core and the heparan sulfate chains. *Free Radic Biol Med.* **49**, 282-293 (2010).
15. Sen, S., Ng, W. P., Kumar, S. Contractility dominates adhesive ligand density in regulating cellular de-adhesion and retraction kinetics. *Ann Biomed Eng.* **39**, 1163-1173 (2011).
16. Wildt, B., Wirtz, D., Searson, P. C. Programmed subcellular release for studying the dynamics of cell detachment. *Nat Methods.* **6**, 211-213 (2009).

