Examining cell-cell interactions in the kidney using AFM single-cell force spectroscopy

Eleftherios Siamantouras¹, Claire E. Hills¹, Kuo-Kang Liu² and Paul E. Squires¹

¹School of Life Sciences, University of Lincoln, LN6 7DL, UK AND ²School of Engineering, University of Warwick, CV4 7AL, UK

Corresponding Author:

Professor Paul E. Squires

School of Life Sciences

Joseph Banks Laboratories

University of Lincoln

Brayford Pool

LN6 7DL

Lincoln, UK

Email: PSquires@lincoln.ac.uk

Tel: 01522886227

Running Head: AFM and cell dynamics

Summary/Abstract:

The ability of individual cells to synchronize activity is a basic feature of efficient and appropriate tissue function, depending on the physicochemical transduction of signaling through multiprotein complexes that functionally mediate adhesion. The direct connection of physical properties and intercellular signaling is of great importance to certain pathologies including diabetes. Atomic force microscopy (AFM) single-cell force spectroscopy (SCFS) is a high-resolution technique that provides a statistically reliable measurement of the minute forces involved in cell tethering and membrane dynamics. Detection of altered nanoscale forces underlying the loss of adhesion in early tubular injury is pivotal for the development of novel therapeutic strategies for diabetic nephropathy. Here we describe the step-by-step use of an integrated AFM-SCFS system designed to measure functional force-displacement in separating renal tubular epithelial cells. Parameters such as unbinding forces, detachment energy and distance to complete separation, can be obtained from force-displacement (F-d) curves and are critical in assessing how physical changes of cellular adhesion contribute to cell contact, coupling and communication in the diabetic kidney.

Key Words: AFM, cell-cell coupling, membrane dynamics, cell adhesion, cadherin, nanomedicine.

1. **Introduction:**

Effective cell-to-cell communication requires contact-dependent intercellular signaling, which relies on the interaction between cells at the adherens junction [1]. Cadherins are calcium-dependent transmembrane proteins that ligate with similar proteins on neighboring cells to form the primary point of cell-cell contact and adhesion in the tubular epithelium [2]. Cadherins have a central role in forming the multi-protein physicochemical pathway that links cell-cell contact to the actin cytoskeleton. This interaction not only increases the adhesive strength of the adherens junction, but also acts as a signalling ‘node’ for proteins that influence adhesiveness and/or initiate intracellular signaling. [3]. Since intercellular signaling requires functional cell-to-cell tethering, monitoring nanoscale changes in physical adhesion, may identify loss of cell function in disease. AFM-based methodologies clarify the intricate relationship between nanoscale physical changes and cellular dynamics, directly connecting biophysics with function. Moreover, application of novel biophysical methods at the medical interface is pivotal in advancing early diagnostics and progressing therapeutic innovations.

Although, AFM-based force spectroscopy was primarily used as an assay to quantify molecular forces between isolated receptor-ligand binding [4], recent advancements in the separation range between cells has allowed the development of single cell force spectroscopy, which provides a description of the ligation function *in-situ* [5-8]. The atomic force microscope (CellHesion® module, JKP Instruments Germany) has a displacement actuator of a long travelling distance (up to 100µm) which facilitates the measurement of complete force-displacement curves when studying the cell-to-cell separation process of large, soft cells, such as found in the tubular epithelium [9]. Retraction force-displacement curves for separating two adherent cells can be measured to determine the force and energy required to

uncouple cells. The former is normally referred to as “maximum unbinding force” F_{max} and the latter the “work or energy of detachment” W_D , which can be calculated from the F-d separation curve [9].

Here, we describe quantitative characterization of the underlying biophysical changes that accompany cell-cell adhesion in the diabetic kidney. Diabetic nephropathy currently represents the leading cause of End Stage Renal Disease in diabetes and is characterized by a number of functional and structural disturbances [10]. Of these, tubulointerstitial fibrosis represents a key underlying pathology and is initiated in response to a loss in epithelial (E)-cadherin mediated cell-to-cell adhesion [11, 12]. Whilst it is well documented that the pro-fibrotic cytokine, transforming growth factor beta 1 (TGF- β 1) reduces E-cadherin expression in tubular epithelial cells of the proximal nephron [13], to further understand how changes in cadherin expression, localization and function directly affect physical adhesion requires the use of sophisticated high-resolution technologies, such as AFM-SCFS. This technique provides a statistically reliable measurement of the minute forces involved in cell tethering and membrane dynamics. In this method, a living cell is attached on a functionalized tip-less cantilever, acting this way as a probe-cell, which is brought in contact with a substrate cell. A laser beam detects the vertical deflection of the cantilever, which is read on a photodetector. The total signal hitting all four quadrants of the photodetector is the Sum output, which is converted in force units during calibration. Any deviation associated with the vertical deflection of the cantilever during cell separation is a measure of molecular binding. This functional measure of cellular adhesion can resolve subtle changes in cell-cell interactions that precede changes in function and can therefore identify underlying mechanisms linking proteomics to overt tissue complications of diabetes. These types of high resolution, reproducible and quantifiable measurements can be used to examine how cell-adhesion and

cell-coupling alter in different tissue types in diabetes. The approach can resolve early physical changes that occur ahead of overt loss in function and are invaluable in understanding the basic cellular changes, which underlie many complications of the disease.

2. Materials:

2.1 Tissue Culturing

1. Clonal Human Kidney (HK2) proximal tubule epithelial cells were purchased from the American Type Culture Collection (ATCC; Gaithersburg, MD 20878 USA).
2. Tissue culture supplies were purchased from Invitrogen (Paisley, UK).
3. Fibronectin was obtained from Sigma (Poole, UK).

2.2 Instrumentation

1. A CellHesion200 module (100µm vertical displacement range, JPK Instruments, Berlin, Germany).
2. Eclipse TE 300 inverted microscope (Nikon, USA) (*see* Note 1).
3. BioCell temperature controller (JPK, Berlin, Germany) (*see* Note 2).
4. CCD camera (DFK 31AF01 Firewire, The Imaging Source, Germany).
5. Anti-vibration table (TMC 63-530, USA).
6. Drivers and Data Processing Software (JPK, Berlin, Germany).

2.3 Probes

1. Tip-less cantilevers (Arrow TL1, 0.03N/m, Nanoworld, Switzerland) (*see* Note 3).
2. Glass block cantilever holder (JPK, Berlin) (*see* Note 4).
3. 2% Hellmanex Solution (Hellma, purchased from Sigma-Aldrich).
4. UV treatment device (Hoefer Scientific).
5. Fine tweezers (BOYA ESD-12).
6. Poly-L-lysine, 25µg/ml (purchased from Sigma-Aldrich).
7. Fibronectin, 20µg/ml (purchased from Sigma-Aldrich).
8. Phosphate Buffered Saline (PBS).
9. Rubber blade cell scraper, manufactured for minimum damage of cells.

10. T25 cell culture flasks.

11. Conical tubes (15ml) for the suspended cells.

3. **Methods:**

AFM-based technology provides a powerful tool for high-resolution, broad range single cell force measurements. SCFS operates on the principal of molecular interaction between a probe cell and a substrate cell. A piezo actuator moves the base of a cantilever towards the surface of a sample cell in vertical direction until contact. After a defined period, the cantilever is retracted while the deflection of a cantilever is continuously measured.

[FIGURE 1 near here]

3.1 Cell Culture

1. HK2 cells (passage 18-30) were maintained in Dulbecco Modified Eagle Medium/Ham's Nutrient Mixture F12 (DMEM/F12), supplemented with 10% fetal calf serum (FCS), glutamine (2mM), epidermal growth factor (EGF; 5ng/ml) and penicillin/streptomycin solution (10,000 units penicillin and 10 mg streptomycin per mL in 0.9% sodium chloride). DMEM/F-12 has a high basal glucose concentration of 17.5 mM to encourage cell growth and promote cell viability.

2. Cells were seeded onto 40mm petri-dishes and cultured at 37°C in a humidified atmosphere of 5% CO₂ in air.

3. Prior to treatment, cells were cultured in DMEM/F12 low glucose (5mM) to negate any pre-stimulatory effects of the high (17.5mM) glucose. Prepared by mixing DMEM (no glucose) and F-12 (10 mM) in a 1:1 ratio.

4. Cells were cultured in low glucose containing un-supplemented (serum-free) DMEM/F12 for 48hr prior to procedure.

5. At this point cells can be treated with a designated stimulus, e.g. TGFβ.

3.2 Sensor Preparation

AFM cantilevers for soft biological materials are very fragile and susceptible to damage. To ensure consistency between measurements, the same type and batch of the cantilevers should be used throughout an experimental project. It is suggested to test the performance of each cantilever before further use, by performing a F-d curve in a hard substrate.

3.2.1 Functionalisation of the cantilevers for cell adhesion experiments

1. Sterilise new cantilevers using UV treatment (5min).
2. Incubate in poly-L-lysine (25µg/ml in PBS) for 30mins at room temperature.
3. Wash cantilevers in fresh PBS.
4. Inspect surface under the optical microscope to ensure smoothness.
5. Incubate in fibronectin solution (20µg/ml in PBS) for 2h at 37°C.
6. Wash cantilevers in fresh PBS.
7. Repeat 4 and 6 if necessary (*see* Note 5).
8. Store in fresh PBS solution at 4°C and use within 3 days. Change the media daily.

3.2.3 Mounting the cantilever on the glass cantilever holder

1. Using a pair of fine tweezers, place the chip of the cantilever on the top of the glass holder.
2. Position the cantilever in the polished part making sure it's aligned towards the middle and in parallel with the alignment marks.
3. Tighten the cantilever on the glass block to ensure that it will be secured into position during experiments.
4. Mount the glass holder in to the AFM head and lock into position (*see* Note 6).

3.3 Instrumentation Set-up

F-d curves are produced by the measurement of the cantilever deflection as a function of the piezo-actuator position. Accurate detection of the cantilever's deflection is crucial as the sensitivity of this operation is directly proportional to the value of the interaction force between the probe-cell and the examining cell.

3.3.1 Laser Path Alignment

Before calibrating the cantilever, we must ensure that the sum voltage output is sufficient. Whenever a new cantilever is mounted on the glass holder the laser path must be aligned, since manual attachment results in slightly different positioning. The laser path is schematically illustrated in figure 2.

1. Adjust the laser spot centrally, towards the end of the cantilever (*see* Note 7).
2. Adjust the prism to ensure that the laser beam hits the region of the photodetector.
3. Adjust the mirror so that the maximum value of output voltage is reached.
4. Fine-tune the quadratic photodetector until the laser hits the centre of it, while ensuring that the lateral and vertical deflection values are close to zero.
5. The sum value should ideally be much higher than 1V and not significantly lower than this value (*see* Note 8).

[FIGURE 2 near here]

3.3.2 Calibration of the Cantilever

For small deflections the cantilevers approximate a Hookean spring, where the deflection is linearly correlated to the acting force. When the cantilever spring constant is known, the

vertical deflection (nm) can be converted into force. Although the nominal force constant value of the cantilever is known, each cantilever must be individually calibrated (*see* Note 9).

1. Set the voltage at 1V (*see* Note 10) and perform a F-d curve on a stiff, clean surface of the petri dish, as it is required to achieve a robust approach for the correct determination of the sensitivity (*see* Note 11).
2. Determine the sensitivity (nm/V) by the gradient of the steep linear part of the curve, close to the repulsive contact region (*see* Note 12).
3. Determine the spring constant of a cantilever using the thermal noise method (*see* Note 13).
4. Fit the function into the chosen peak.
5. Select the appropriate correction factor.

3.4 Experimental

3.4.1 Preparation of Suspended Cells - Scrape Delivery Procedure

This procedure takes place before each treatment is tested.

1. Treat the cells in a T25 flask (*see* Note 14)
2. Gently scrape the cells off the flask's surface using a sterile rubber blade cell scraper with low-force sweeping motion.
3. Transfer the scraped cells using a pipette in a sterile conical tube for centrifugation (5min, 1000rpm, RT).
4. Remove the supernatant and carefully re-suspend the cell pellet with fresh sterile DMEM CO₂ free media (2-3mL depending on pellet size) (*see* Note 15)
5. Gently agitate the pellet using a 1000μL pipette (3min), to break up the cells and ensure a uniform distribution.
6. Incubate for 5min at 37°C to allow recovery (*see* Note 16).

7. Identify a clean area on the petri dish (*see* Note 17) and introduce 20-30 μ L of re-suspended cells with gentle force to ensure fluid movement is kept to a minimum (*see* Note 18).
8. Carefully place the AFM head into the stage and proceed to the next step.

3.4.2 Single Cell Attachment Procedure

The attachment procedure must be performed rapidly (2-5min), otherwise suspended cells will adhere on the substrate.

1. Set the AFM in constant force mode.
2. Set the force at 1nN, the contact time at 5sec and the speed at 5 μ m/sec (*see* Note 19).
3. Identify a suitable cell (*see* Note 20) and place the arrow shape cantilever directly above the cell.
4. Perform a F-d curve.
5. Once contact is established, gently position the cantilever so that the cell is attached towards the apex.
6. Inspect the attached cell and leave to recover for at least 5min.
7. To ensure that rigid adhesion of the probe-cell is achieved, move the cantilever in the medium while observing the attached cell.

[FIGURE 3 near here]

3.4.3 Cell-to-cell adhesion

1. Set the contact force at 1nN, contact time at 10sec and constant speed at 5 μ m/sec (*see* Note 21).

2. Identify a substrate cell and position the probe cell directly on top of nucleus (*see* Note 22).
3. Perform a F-d curve.
4. Check the displacement output to ensure that the piezo is functioning in its effective range while being able to touch the cell and completely separate the cells when retracted.
5. Adjust the piezo if necessary or skip to 6.
6. Allow the cells to recover for 30sec at the maximal retraction distance of the piezo.
7. Perform F-d x 2 on the same cells, with 30 sec intervals between measurements.
8. Allow the probe cell to recover for 1min between readings.
9. Move the cantilever gently to the next substrate cell, follow steps 3-6 (*see* Note 23).
10. Perform experiments in 10 cells using the same probe-cell (30 F-d curves/dish).

[FIGURE 4 near here]

3.4.4 Retraction Curve analysis

1. Open the batch function of JPK's Data Processing.
2. Load the curves using the empty-process selection.
3. Select the function to automatically subtract the baseline.
4. Select the retract segment.
5. Select the linear part of the F-d curve on the x-axis.
6. Select the option Offset plus tilt if necessary.
7. Select the Measure Adhesion function to determine the maximum unbinding force.
8. Select the Determine the area under the curve function to calculate the work or energy of complete detachment.

9. Select the Identify and quantify steps function (*see* Note 24).
10. Select Only Positive Steps (*see* Note 25).
11. Select the area of the retraction curve to fit the step-fitting model (*see* Note 26).
12. Run the automated step-fitting method to determine the tether rupture events.

[FIGURE 5 near here]

4. Notes:

1. It is necessary to install the force spectroscopy module on an inverted microscope to maintain optical view in order to effectively manipulate the cells.
2. The temperature controller should be adjusted two degrees higher than the desired temperature to ensure that the sample is maintained at 37°C to achieve near-physiological conditions.
3. The selection of the parameters of the cantilever depends on the type of cells and it should have a low spring constant when the sample cells are from soft tissues/organs. In addition, the arrow geometry of the cantilever facilitates the manipulation and capture of the probe cell.
4. This specially designed cantilever holder is polished on its top and bottom surfaces, to allow transmission of light to the lenses and therefore extra care should be taken to avoid scratching, hence they were not touched with hands or tweezers. Cantilevers can be cleaned in an ultrasonic bath using a floating rack and mild detergent.
5. Since the output sum signal depends on deflection of the laser, it is crucial to ensure that the surface of the cantilever is clean and any residual debris should be removed from the cantilever after functionalization.

6. The way the cantilever is mounted affects the sensitivity. If the output reading is faulty, re-mounting the cantilever may troubleshoot.
7. The illumination of microscope can be decreased to ease the view of the laser on to the cantilever.
8. A relatively stable sum value (Volts) should be generated by the vertical and lateral deflection of the cantilever, however if the experiments are conducted in culturing media a small drift may appear. If, after the proper alignment of the laser, the Sum value is significantly below 1V or if there's high drift in the deflection of the cantilever, then a new cantilever or fresh media should be used. When using chemically modified cantilevers, it is important to use a clean region of the cantilever as the deflection site.
9. A calibrated cantilever must be re-calibrated if the alignment parameters are changed or if it loosely attached on the glass holder.
10. Higher values may compromise functionalisation. Approach curves at this stage should be kept to a minimum.
11. It is crucial that this measurement is performed in the same buffer solution and temperature as in the actual experiments. Ideally, there should be space on the cultured dish, depending on the confluency, however if this is not possible a clean dish with the same experimental conditions can be used.
12. This is the distance of the cantilever vertical deflection for a given Voltage difference measured by the photodetector and by calculating sensitivity, deflection can then be converted in to distance (nm). Typical sensitivity ranges are between 30-100nm/V, as stated by the manufacturer. Sensitivity depends on the reflective characteristics of cantilever, the mounting position of the cantilever and should be determined every time the cantilever is mounted or re-mounted [6].

13. For a given temperature, the amplitudes of the fluctuations depend only on the spring constant of the cantilever; hence the thermal resonance curve can be fitted to a Lorentz function to allow spring constant calculation [14]. However, the resonance of soft cantilevers in culture media is much lower and very susceptible to noise due to drifting of the cantilever, in which case a correction factor should be used to calculate the actual value of the spring constant [15]. Also, when using cantilevers for soft biological material in liquid the first resonant peak is largely affected by low frequency noise. In that case, the second peak and a correction factor of 0.251 should be used. To increase consistency between measurements, it is suggested that cantilevers from the same batch should be used.

14. Using a flask to culture 'free' cells reflects ease of accessibility when detaching cells with a scraper.

15. Experiments were performed in an instrument that is not equipped with a CO₂ chamber, hence the use of CO₂ independent media.

16. At this stage please proceed with 1 and 2 of next paragraph.

17. Perform an approach curve to adjust displacement into the piezo range prior to pipetting suspended cells. Retract the piezo (100µm) before lifting the head.

18. Gently agitate 'free' cells in the tube with a 1mL pipette to re-suspend any pelleted cells (10sec) before introducing them to the dish. To avoid fluid and particle movements, it is important to limit the amount of 'free' cells entering dish.

19. Attachment force and contact time may need to be slightly adjusted between experiments, the ranges we suggest are 0.8-1.3nN and 5-10sec.

20. Ideally should be a cell with a rounded shape, smooth surface and comparable diameter with the other suspended cells floating in the media. If the identified cell is relatively stable on the substrate, gently touch the head with a finger and observe whether or not the cell is affected by the fluid's motion. If not, it may be already adhered to the substrate.

21. Complete separation depends on contact time, contact force and retraction speed. These parameters were adjusted before experiments to ensure that the maximum piezo range is sufficient for complete separation.
22. Since there is a difference in height between substrate and probe cell, fine adjustment of the lens is required to identify the exact position of contact.
23. It is advised to explore the substrate cells prior experiments, to identify areas of potential testing cells in close proximity.
24. Unbinding of ligations can be detected by identifying sharp steps of force that occur after a main rupture of tethers and a plateau in x-axis that is dependent on cell compliance. The software is based on the work of Kerssemakers et al. 2006 [16] and works well as a step-fitting method for detecting small force jumps that correspond to bond rupture. However, the automated analysis is not fully developed and some events may not be detected. Nevertheless, differences in the molecular ligation between treatments can be observed.
25. During the retraction phase only positive steps are anticipated.
26. Zoom in the curve to determine the exact position of fitting.

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Figure Legends:

Figure 1: A long-range piezoelectric actuator controls the position of the cantilever while any changes in deflection are detected by a quadratic photodetector. This arrangement allows high-resolution continuous measurements of force versus displacement during the separation process of two cells. In Phase A, ligation is formed when the cells are in contact and in Phase B surface molecules are unbound during the separation process.

Figure 2. Optical path of the laser: By adjusting both the mirror and the photodetector the laser spot from the end of the cantilever must be targeted to the centre of the photodetector to achieve maximum sensitivity (Figure adopted from JPK's manual).

Figure 3A. A suitable suspended (free) cell is identified and attached on to the functionalised cantilever. B. The probe (attached) cell is then positioned over a substrate cell within a cell cluster, before the cantilever is lowered and the attached and substrate cells allowed to adhere under constant force (1nN/10sec).

Figure 4. A typical force distance curve generated by the coupling and uncoupling of two cells. Initially the cells are bounded into a cantilever and the substrate (phase 1). Then the cells are made to 'approach' each other. After a short period of contact, the two cells adhere. During this 10s period bonding is formed (phase 2). Next, the cantilever is retracted and force versus displacement measured (phase 3) until the cells are completely detached (phase 4).

Figure 5. Raw F - d cell-to-cell adhesion curves. All processing functions were applied to the retracted F - d curve. (a) The reference point that will be used for the determination of the

adhesion parameters, such as F_{max} , and W_D should be determined. Essentially this is the point of complete separation between the two cells and is specified by setting the baseline of the complete separation of the retraction curve to match the zero x-axis. (b) The detection of minimum value of force represents the maximum force required for the complete separation of the two cells. F_{max} is calculated by detecting the minimum negative value of force with respect to the x-axis baseline. By determining the area under the retracted part of the curve, work or energy of complete detachment W_D was calculated. (c) The retraction curve clearly exhibits a step-like pattern as an immediate result from the rupture of surface ligations. Tether rupture events can be detected by identifying sharp steps of force that occur after unbinding of ligations. These events fall into two areas, those in which a ramp in the deflection of the cantilever is preceded and those in which a mechanical deformation is preceded as the pulling distance increases.