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Nanoscale characterization of forensically relevant epithelial cells and surface associated extracellular DNA

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ABSTRACT

Atomic force microscopy provides a novel morphological and physico-chemical perspective to analyze epithelial cell samples in forensic investigations. As a nanoscale, single cell tool, it allows the investigation of scarce samples in a non-destructive fashion. Using chemical force spectroscopy, it permits the identification of specific functional groups or surface molecules. Of specific interest is the presence of extracellular DNA (*e*DNA) on the surface of epithelial cells that line the exterior skin and interior cavities of human bodies, and can transfer onto surfaces through contact with skin and saliva. To date, this *e*DNA has only been measured a bulk level. Here, using nanoscale imaging, we first describe the unique differences between keratinized epithelial cells and non-keratinized buccal cells. Then *via* a force mapping technique, we show how *e*DNA can be spatially located and quantified on the cell surface. Our results suggest that presence and relative quantity of surface-associated, extracellular DNA signatures can be analyzed on individual epithelial cells from different tissue sources, providing a new tool in the forensic analysis of touch samples.

1. Introduction

Epithelial cells of various types line the exterior skin and interior cavities of human bodies. In the case of skin, the outermost layer is composed of keratinized, stratified squamous epithelial cells. In contrast, tissues that line the inside of the mouth, or the esophagus are composed of non-keratinized, stratified squamous epithelial cells [1]. Both kinds of epithelial cells are important in various forensic contexts owing to cellular and DNA transfer onto surfaces through contact with skin and saliva, respectively [2–4]. In many cases, the limited quantity of such cell samples often makes it difficult to conduct bulk scale analyses. Therefore, tools that can probe epithelial cell samples at the level of a single or few cells are needed for analysis and identification [5]. To date, while several images have been obtained by staining and optical microscopy [6], the nanoscale surface characteristics of the cells have yet to be elucidated.

An additional factor of forensic interest on the surface of epithelial cells (palm and buccal) is the role of extracellular DNA (*e*DNA). For many cell types, some portion of extracellular DNA is physically attached to the outer leaflet of the plasma membrane [7]. These cell membrane-associated *e*DNAs originate at the cell nucleus, are expressed at the cell membrane and remain attached to it. Extracellular DNA can also exist unattached to the cell surface, freely circulating within bodily fluids or secretions such as sweat, oil, or blood plasma. These circulating 'cell-free' DNA molecules likely result from cellular apoptosis [8]. While the exact function of the *e*DNA is not known, several recent studies are establishing their forensic as well as biomedical importance. The concentration of *e*DNA has been correlated to some pathologies including cancers and autoimmune diseases [9]. *e*DNAs can serve as specific targets for IgG autoantibodies in systemic lupus erythematosus patients [10]. They have also been postulated to have serve key roles in cellular adhesion and mechanical stability of biofilms [11].

A number of studies have found that extracellular DNA can be a significant component of epithelial cell samples [12]. For 'touch' or contact samples in particular, which are composed almost entirely of cells from the outermost layer of skin, the *stratum corneum*, *e*DNA may constitute an overwhelming majority of the total recoverable DNA in a touch sample. Yet, many biologically relevant aspects of *e*DNA and the relationship to co-deposited epithelial cells are currently unclear. Detecting and mapping the extracellular DNA on epithelial cells, including the relative contributions of loosely-bound and surface-associated DNA could provide a unique

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look at its structure and spatial/biochemical context. This has potentially important implications for understanding the mechanisms of DNA transfer through touch, maximizing the recovery of DNA from contact surfaces, as well as understanding its role in various biological and forensically-relevant phenomena.

The goal of this study is to study the presence and relative quantity of extracellular DNA on two forensically relevant epithelial cell types: the non-keratinized buccal cells and the keratizined epidermal cells derived from the palm and fingers. Initially, we present images of these cell types to understand their nanoscale topography. Then, we present spatial maps showing the distribution of *e*DNA and comparison across each cell type using samples obtained from different individuals. The tool for these studies is the atomic force microscope (AFM). Over the last few decades, the AFM has emerged as one of the most powerful and widely used tools for high precision imaging and sophisticated biophysical investigations [13–15] and recently, for forensic investigations [16,17]. It is a versatile tool to investigate samples due to simple and rapid sample preparation, and measurements under near-physiological conditions [18,19]. Importantly, as a nanoscale technique, it allows non-destructive and real-time studies of sample populations containing only a few cells.

To understand the surface distribution of *e*DNA across cell types, we utilize a novel mapping strategy that involves probing the cell surface with AFM cantilevers functionalized with a lactoferrin probe, a protein that specifically binds to cell-membrane DNA [20]. For the first time, we demonstrate spatial detection of *e*DNA non-destructively and at the single cell level. As part of this study, the prevalence of surface-associated *e*DNA was also examined across multiple sample washing steps to elucidate the nature of the attachment between *e*DNA and the cell surface. By estimating binding percentages of the probe to surface DNA, it is interesting to observe differences across donors. As a control, the identical cells were imaged before and after (1 h) the addition of DNase. The significant decrease of binding percentage further confirms the specificity of the lactoferrin probe, and the cell surface DNA interactions. Thus, the reported cell surface DNA profiling could be a potential tool for forensic identification at the single cell level.

2. Experimental

2.1. Materials

(1-Mercaptoundec-11-yl) hexaethylene glycol (Oligoethylene glycol (OEG) terminated thiol), HS-C_{11}-$(EG)_6$-OH, and (1-mercaptohexadecanoic acid)-*N*-succinimidyl ester (NHS terminated thiol), HS-C_{15}COO-NHS, were purchased from Nanocs Inc. (Boston, MA). Lactoferrin and DNase II were purchased from Sigma–Aldrich (St. Louis, MO). Phosphate-buffered saline (PBS pH 7.4) (11.9 mM phosphates, 137 mM sodium chloride and 2.7 mM potassium chloride) and ethanol (200-proof) were purchased from Fisher Scientific (Waltham, MA). Mica was purchased from Ted Pella (Redding, CA). Ultrapure water (resistivity 18.2 MΩ cm) was obtained from a MilliQ water purification system (Millipore Scientific, MA). AC240TS cantilevers (Olympus) were used for non-contact mode imaging in air, while gold coated TR800PB cantilevers (Olympus) were used for force measurements.

2.2. Cell collection

Epithelial cell samples were collected from volunteers using Institutional Review Board (IRB) approved protocol (HM20000454_CR3). Buccal cells sampled by having individual rub the inside of both cheeks with a sterile pre-wetted cotton swab (Part number: 22037924; Fisher Scientific). Cells were eluted by placing the swab in 1× PBS and vortexing for 15–20 s. For touch epidermal cells, individuals lightly gripped a sterile polypropylene conical tube (Part number: 229421; Celltreat Scientific) with their palm and fingers for 5 min. Cells were collected from the surface using a wetted cotton swab and eluted in 1× PBS as before. For experiments involving washing steps, cell solutions were first centrifuged at 3000×*g* for five minutes washed cells. The supernatant was removed from the cell pellet either by decanting or pipetting and then re-dissolved in 500 μl of sterile 1× PBS. To examine the effect of sample drying and/or aging, a subset of samples from each cell type was dried onto a glass slide and left at room temperature in a biological safety cabinet for 3, 7, and 21 days.

2.3. Sample preparation for AFM

Cells were immobilized using a poly-L-lysine fixation method [21]. Mica was coated with poly-L-lysine to increase the adherence of the cells. Freshly cleaved mica was immersed for 10 min in a solution of 0.05 mg/ml poly-L-lysine hydrobromide and 10 mM Tris (pH 8.0). The surface was then covered and dried vertically overnight at room temperature. The coated mica was stored at room temperature and was used within one week. Concentrated cells suspended in PBS were deposited on the coated mica and incubated for 30 min. The cells were kept hydrated at all times. Excess cells were rinsed off with three washes of 1 ml water. Images were taken in PBS using non-contact mode imaging. To observe the cells *in situ* and in real time, the same cells were imaged before and after (1 h) addition of DNase (control experiment).

2.4. AFM probe functionalization and force recognition mapping

AC240TS cantilevers were cleaned using high-intensity UV light to remove organic contamination and used for imaging in air and characterization of the surfaces in non contact mode. Gold coated cantilevers were cleaned in UV/ozone for 15 min and then functionalized by immersion in mixed thiol solution (HS-C_{11}-$(EG)_6$-OH and HS-C_{15}COO-NHS) in ethanol for 16 h [22]. Cantilevers were rinsed with ethanol, and incubated in a 100 nM lactoferrin in PBS buffer for 1 h at ambient temperature. This functionalization strategy limits the number of proteins on the tip surface to 1–3 [23]. Lactoferrin-functionalized cantilevers (spring constant ~0.15 Newton/meter (N/m), resonance frequency 15 kHz) were used to obtain force data on cells in PBS. Each experiment was repeated on at least 3 different cells. Force curves were obtained by collecting a series of sequential force curves in an $m \times n$ grid (an array with m lines and n points per line). Each force curve was obtained at the same loading rate (135 nN/s), allowing binding to occur (contact time 0.1 s), and then retracting. 50 × 50 force curves were collected over a defined area and unbinding forces were displayed on a scale of color. The height maps of the same area were generated simultaneously. As a control, DNase experiments were performed by a buffer exchange of PBS containing 100 μg/ml DNase in the fluid cell. All images including height and force maps were analyzed using custom routines in Igor Pro 6.32 A (Wavemetrics Inc, OR).

3. Results and discussion

The application of atomic force microscopy with its capabilities of high resolution imaging and force spectroscopy, with forensic science, provides an exciting perspective to study cells [24,25]. Importantly, the AFM can directly investigate only a few cells (trace samples) either in air or under near physiological environments in a non-destructive fashion. Force spectroscopy for