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Final Report
Nanoscale Imaging and Chemical Analysis of Extracellular DNA in Trace Evidence Samples

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Date

Summary of the project

Major goals and objectives

Analysis of trace cellular evidence has become an integral part of a forensic laboratory's workload, and an important tool for investigators. The outer surfaces of epithelial cells hold the key to answering many fundamental forensic questions. However, the relationship between the genetic profile from a touched object and the proportion of DNA that is cell-free/extracellular, or is associated with epidermal cells themselves has not been fully elucidated. The objective of this project was to advance the fundamental and applied science of understanding epithelial cell surfaces for forensic analysis. Using nanoscale (e.g. atomic force microscopy) and traditional analytical tools (e.g. flow cytometry), we proposed to probe cells for their extracellular, cell-surface attached DNA (eDNA). Specifically, our objective was to understand the physical and chemical nature of trace cell deposits transferred via touch or contact. This included the differences between various cell types, as well as the spatial and biochemical context of DNA on the cell surface. Using high resolution, non-destructive tools we proposed a unique look at the cell surface – its nanoscale morphology, its mechanical properties and the presence of specific cell surface signatures including eDNA. These spatial and temporal signatures will lead to a better understanding and handling of cellular samples collected as evidence, while developing ultrasensitive tools for cell attribution, and precise analysis of touch and contact DNA. The aims of the project were to:

1. Characterize trace cell populations deposited through touch/contact using single cell techniques in order to identify unique morphological and nanomechanical signatures of epithelial cells derived from different tissues.

2. Probe the cell surfaces to locate macromolecular biomarkers including eDNA and characterize

its relationship to larger cell population and total DNA content of the sample

Research questions

The specific scientific questions were as follows:

Do epithelial cells from different sources exhibit physical differences in the structure, biochemical, and mechanical properties of their outer membrane?

What is the spatial and temporal distribution of *e*DNA on the surface of cells? Is this correlated to the total DNA yield from a biological sample? How does this signature change across cells?

Can this knowledge be used on the front end of an operational DNA workflow to quickly and non-destructively predict which samples are likely to provide probative DNA profiles?

Research design, methods, analytical and data analysis techniques

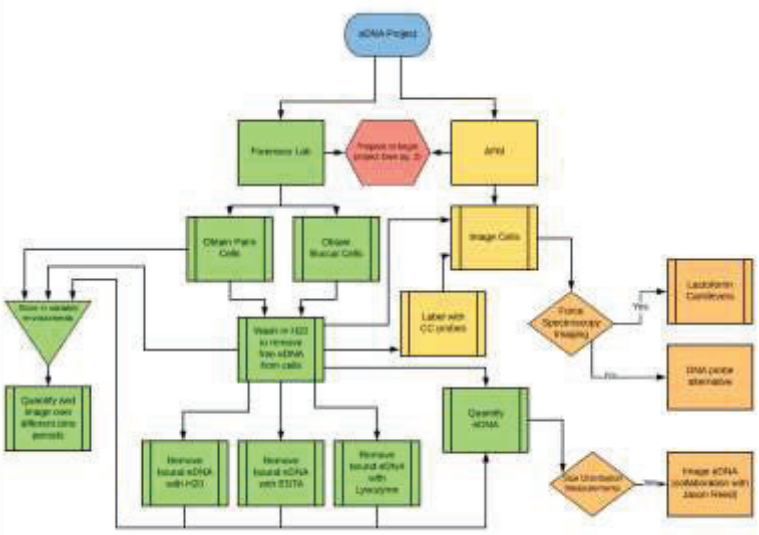

Figure 1 – Research design and proposed work flow for the project.

The research design in this project consists of a multidisciplinary integration of single cell techniques (atomic force microscopy) with ensemble techniques (flow cytometry) to obtain a unique perspective on cell-associated extracellular DNA (eDNA). The eDNA is obtained from a sample set of human donors, which further provides an opportunity to

conduct a survey on the quantitation and variation of shedding across individuals. The broader workflow is shown in Figure 1. Briefly, it consisted of the following:

1. Characterization of trace cell samples using ensemble and single cell techniques - The objective of this step is to identify morphological, biochemical, and nanomechanical signatures of epidermal cells. These experiments will result in a unique database of cell types typically collected as evidentiary samples – offering a clear visualization of cell morphology, their biophysical properties, and biochemical signatures to provide confirmatory analysis on cell type and source.

2. Probing the cell surface to spatially locate and visualize macromolecular biomarkers including extracellular DNA - The focus on the eDNA aspects of cell surfaces using surface biomarkers that will provide probative data for evidentiary samples.

3. Correlation between extracellular DNA and total genomic content - While the goal of the previous tasks is to characterize cellular and biochemical signatures of cell types and eDNA, we will test how the signatures obtained correlate to bulk genetic properties of a touch sample (DNA yield, STR profile quality) as measured through conventional forensic molecular techniques.

Taken together, these experiments will result in a fundamental understanding of mechanisms of attachment and distribution of eDNA on cell surfaces.

Expected applicability of the research

This research has led to strategies to optimize the recovery and profiling of trace DNA from an aged, degraded, and/or low template sample, by providing an understanding of the source (epithelial cells) and nature of DNA (whether it is free or anchored to the surface) transferred through touch or contact. To date, these interdisciplinary biophysical and biochemical tools have not been used in the forensic arena. The direct outcome of this project is methodologies for rapid,

ultrasensitive and non-destructive sample analysis in forensic applications and an enhanced understanding of key biological properties of extracellular DNA and cell types relevant to forensic casework. This research provides interdisciplinary and nanoscale perspective to cellular evidence analysis. The number of touch and contact samples being collected and submitted to case working units remains a problem, and better ways of not only handling such evidence, but understanding the fundamental science behind them are needed. Often, investigators have to work with limited amounts of cells and/or DNA, underscoring the need for new kinds of ultrasensitive tools that can also be rapid and reliable.

Participants and other collaborating organizations

Researcher training has been a significant component of this project. These have been undergraduate students (Mekhi Miller, Zulhumar Adil, Jessica Daniels), post-bac student (Emily Brocato), graduate student researcher (Kristin Jones) and a postdoctoral associate (Dr. Anita Olsen). A post-graduate technician (Mary Tootham) was also involved in the project. These researchers worked on both fundamental techniques – e.g. forensic DNA extraction techniques and quantification, PCR amplification/quantitation, flow cytometry, and imaging flow cytometry analysis. Atomic force microscopy and fluorescence imaging was primarily performed by the postdoctoral associate and the PI.

The multidisciplinary nature of this project has translated to researchers being trained in diverse topics pertaining to the applications of biology and nanoscience in the forensic field. Most notably, the participants in this project were able to contribute to the technical papers that resulted.

No other organizations participated in this project.

Changes in approach from original design and reason for change, if applicable

Overall, this project has been very successful in terms of delivering on the proposed specific aims and milestones (see output below). There have been minor changes in the original design of the proposed research, specifically with regard to investigating aged samples. Some adjustments had to be made at the start of the project (Mar 2018 – Sep 2018) due to personnel hiring turnover issues, which led to delays in starting work. Similarly, at the back end of the project (Mar 2020 – Jun 2020), researcher turnover and the COVID-19 pandemic led to a suspension of laboratory research. Fortunately, we were able to complete data analysis and manuscript preparation during this period. Both of these issues led to an under-spend on the budget which has been returned to the agency. However, it may be noted that we have been able to fulfil the deliverables on the project.

Outcomes

Our activities and results from this project can be classified along three broad outlines:

1. Development of an automated workflow for the detection and characterization of epithelial cells from trace biological samples

Forensic laboratories are often dealing with large numbers of cell samples collected. In order to streamline this process, we have devised an unsupervised workflow for identifying epithelial cells in microscopic images, and characterizing their morphological and/or optical properties. The proposed method can be used on cells that have been stained with fluorescent dyes and imaged using conventional optical microscopes. Based on donor derived cells, the workflow was tested on cell populations that were imaged directly on touch/contact surfaces and stained with nucleic acid dyes to visualize genetic content. Epithelial cell samples were collected from 15 individuals

following Institutional Review Board (IRB) approved protocol (ID# HM20000454_CR5). Touch epidermal cells were obtained by having individuals press their finger or thumb directly onto a glass slide surface for 10 seconds. For nucleic acid staining, GelGreen® Dye was used. By staining the cells with fluorescence dyes, it was possible to quantify fluorescence on a per cell basis. Open source software was used (viz. CellProfiler) making this an easily accessible, rapid, low-cost analysis tool (Figure 2).

Our results show that this approach could be a useful strategy for characterizing differences in staining efficiency, and/or morphological properties of individual cells, and even differentiate contributor cell populations within a trace deposit in an automated fashion. This can also potentially reduce the laborious nature of microscopic analysis and increase throughput and reproducibility of similar basic research studies. This portion of the project was published during the last phase of the project (Olsen et al., 2020a; See Dissemination section below)

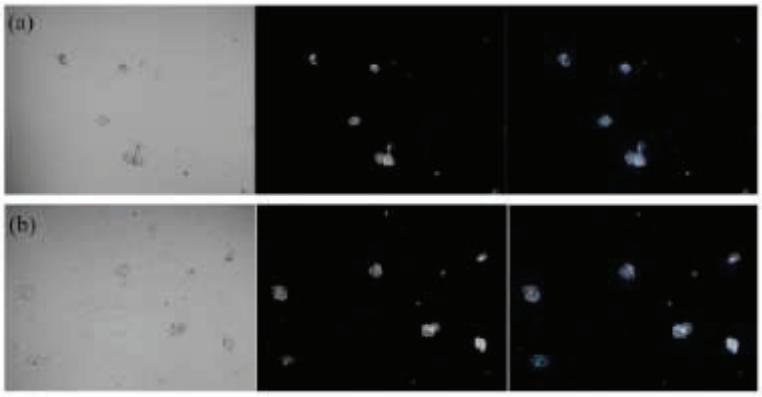

Figure 2 - Image processing workflow for cell populations from Individuals 6 (a) and 15 (b). Brightfield and fluorescence images are shown for the same field of view (left and middle panels respectively). Segmentation scripts are then applied to fluorescence images to detect individual cells and extract measurements (right panel).

2. Survey of extracellular DNA abundance in touch samples

Using the techniques developed, we were able to conduct a survey of extracellular DNA abundance in touch samples and quantify the potential attachment mechanism to the cell surface, which was

one of the key deliverables of this project. The quantity of eDNA and its proportion to the total genomic content within trace epithelial samples was obtained from 24 different individuals. DNA yields in the extracellular fraction showed wide variation between donors ranging between 0 and ~11 ng. In contrast, DNA yields from the remaining cell pellet were fairly consistent between individuals, ranging between 0 and 0.228 ng. There were no obvious correlations between samples with higher amounts of pellet-associated DNA (e.g. > 100 pg) and higher yields of eDNA (e.g. > 1 ng). Despite the high variability across individuals, samples showed relatively high proportions of eDNA (>80%). Samples with lower eDNA proportions showed total DNA yields < 200 pg (Study currently under review at Forensic Science International).

To investigate the extent to which the abundance of extracellular and cell-pellet DNA varies between trace deposits left by the same contributor, multiple touch samples were collected from nine donors on different days. The results showed that eDNA yields varied widely across replicates for the same individual, with ten (or more)-fold differences typically observed between highest and lowest yield deposits. The wide variability both between individuals and across replicate samples deposited by the same individual indicate that eDNA deposition is likely influenced more by extrinsic factors such as activities prior to deposition rather than intrinsic/genetic characteristics of the donor. Conversely, DNA yields from pellet associated fraction were remarkably consistent across all donors and across replicates from the same donor (0-200 ng), indicating that this fraction does not contribute to variation in recoverable DNA from touch samples.

One interesting implication of the low levels of DNA observed in the cell pellet together with the absence of nucleated cell types, is for front-end cell sorting efforts which necessitate the presence of cells with sufficient amounts of intracellular DNA so that probative profiles can be obtained after sorting. The prevalence of eDNA in trace samples may require novel approaches for sorting

trace cell populations, and/or coupling information from both the eDNA and cell-pellet fraction to be successful. Determining the relative proportion of eDNA versus intracellular DNA has important implications for ongoing efforts to develop methods for isolating contributor cell populations from trace biological mixtures for single source DNA profiling.

3. Development of novel imaging tools to visualize extracellular DNA on surfaces

We have advanced the development of tools to visualize the eDNA on the surface of epithelial cells. In the above results, we were able to show how data from several donors could be analyzed at the ensemble level. High resolution atomic force microscopy (AFM) was employed to obtain a look at these epithelial touch samples at the single cell level (Figure 3). We further demonstrated the integration of optical microscopy with AFM for the visualization of topographical distribution of eDNA using fluorescence imaging (Figure 4). We showed how a surface-attached target can be interrogated using this strategy, specifically providing a direct visualization of the surface-attached eDNA. Different nucleic acid dyes (Gel Green, Diamond™ Dye) were compared in order to suggest an optimal dye based on intensity and ease of use in order to study such samples. Epithelial cells obtained from touch samples were labeled with a DNA-specific fluorescence probe and the resulting images were overlaid on topographical images to show the 3D distributions and concentrations of DNA on or within the cell. Fluorescence intensities were compared between cells with and without DNase treatment, that removes any eDNA from the surface of the cell. These results utilized micro and nanoscale measurements to identify and elucidate attachment

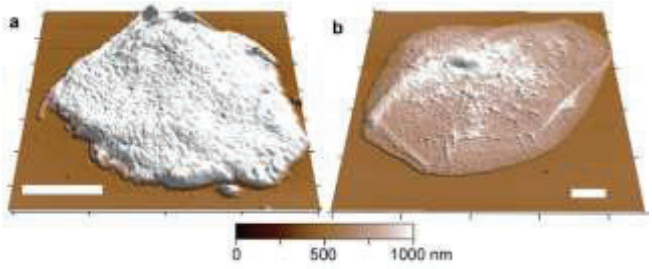

Figure 3 - High resolution nanoscale imaging of epithelial cells (from (a) palm and (b) cheek)

mechanisms of eDNA.

They can also assist in bridging the gap between single cell analysis conducted with AFM and bulk studies such as qPCR and flow cytometry (used for the automated workflow discussed above). Optical fluorescence microscopy and flow cytometry both give two-dimensional shape and fluorescence information that were directly correlated. DNA quantification from sample washes and cell pellets are combined with cell concentration data to determine the distribution of intra- and extra-cellular DNA within samples. The results can then be compared between donors and treatment with AFM/optical microscopy of individual cells to observe variance and behavior of eDNA within touch samples. Taken together, these results provide a new insight into collection of "touch" cell samples, handling them (washing) in order to retain/obtain cell-surface eDNA, and finally to visualize the eDNA either directly on the cell or quantitatively. Results for these experiments were published in the last phase of the project (Olsen et al., 2020b; See Dissemination section below).

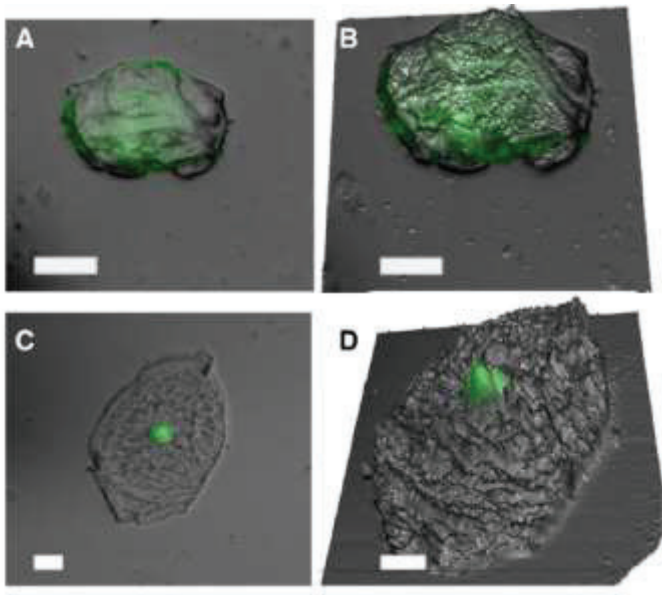

Figure 4 – 3D overlay of the fluorescence image with the AFM image showing the spatial location of the extracellular DNA on epithelial samples. Panel (A) shows palm cells whereas panel (C) shows buccal cell samples. Images are overlaid on the 3D topography (B), (D) respectively, providing a unique look at the cell. Scale bars = 10 μm on all panels.

Our work and experiences with the atomic force microscopy and related nanoscale techniques was summarized into a review covering the multimodal approaches and nanoscale capabilities that may be used by forensic investigators.

Limitations

The methods developed for this project were demonstrated on a variety of cell types (buccal, shed epidermal) and sample conditions (dried for several days). However, they have not been tested on samples that have been more significantly aged/degraded as is common in DNA casework. An additional area of future research would be to examine the effect of various storage conditions on the integrity of nanomechanical and fluorescence signatures as well as the proportion of cell-free and cell-associated DNA yields. This may lead to better understanding of physical and temporal controls on these signatures and potentially improved protocols for analyzing samples approaching those encountered in forensic laboratories.

List of products (e.g., publications, conference papers, technologies, websites, databases):

1. Olsen, A., Miller, M., Yadavalli, V. K., & Ehrhardt, C. J. Open source software tool for the automated detection and characterization of epithelial cells from trace biological samples. Forensic Science International, 110300, 2020a https://www.sciencedirect.com/science/article/pii/S0379073820301626?dgcid=rss_sd_all

2. Olsen, A., Ehrhardt, C. J., & Yadavalli, V. K. Nanoscale visualization of extracellular DNA on cell surfaces. Analytical Science Advances, 2020b (*open access*) https://onlinelibrary.wiley.com/doi/full/10.1002/ansa.202000095

3. Tootham, M., Miller, M., Yadavalli, V. K., Ehrhardt, C. J. Survey of extracellular and cell-pellet-associated DNA from 'touch'/trace samples. Forensic Science International, *under revision*, 2020.

4. Yadavalli V.K, & Ehrhardt, C.J. Atomic Force Microscopy as a biophysical tool for nanoscale

forensic investigations. Science & Justice, *under review*, 2020

Data sets generated

Information corresponding to publication #1 above is in an open access repository – bioRxiv 2020.03.24.006296; doi: 10.1101/2020.03.24.006296

Dissemination activities

1. "Nanoscale Imaging and Chemical Analysis of Extracellular DNA in Trace Biological Samples" presented at the Academy's 71st Annual Scientific Meeting - February 18-23, 2019, in Baltimore, MD, USA.

2. "Nanoscale Imaging and Chemical Analysis of Extracellular DNA in Trace Biological Samples" presented at the NIJ poster session at Pittcon 2019 - March 17-21, 2019, in Philadelphia, PA, USA. The copy of the poster has been uploaded to the figshare data repository: doi: 10.6084/m9.figshare.8983565.v1

3. Mekhi Miller presented results from bench experiments at the Undergraduate Research Symposium held at VCU in May 2019.

4. Our group participated in two outreach activities - Full STEAM Ahead (June 29, 2019) dedicated to empowering young women through Science, Technology, Engineering, the Arts, and Mathematics. Dr. Olsen and another member of our group presented nanoscale techniques in the sciences to middle school girls, (6th through 9th graders), in the Greater Richmond Area.

NanoDay at the Science Museum of Virginia (October 5, 2019). This half-day-long event was available to the general public in the Greater Richmond Area. Members of our group presented demonstrations at this event to show nanoscale techniques in the sciences including the use of atomic force microscopy for forensic applications.