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Final Technical Report

DNA Profiling of Complex Biological Mixtures using HLA-Antibody Probes and Fluorescence Activated Cell Sorting

Award # 2013-DN-BX-K033

Author: Christopher J. Ehrhardt

Abstract:

Analysis of biological mixtures is a significant problem for forensic laboratories. The presence of cells from multiple individuals in a biologic stain complicates DNA profile interpretation and often leads to loss of evidence. While many analytical techniques have been developed to address complex STR profiles resulting from cell mixtures, most are optimized for separation of sperm from epithelial cells and few can be applied to biological mixtures containing one cell type. Yet, an increasing proportion of samples submitted as evidence are contact epithelial mixtures, in which there are no physical differences between cells contributed by multiple individuals. New techniques are needed to separate individual cell populations from these types of forensic mixtures to generate unambiguous STR profiles.

The goal of this project is to develop a new analytical technique that utilizes the intrinsic immunological variation among individuals to physically separate cells from different sources prior to DNA profiling. Specifically, we have used fluorescent antibody probes targeting two protein classes of epithelial cells, Human Leukocyte Antigens (HLA) and Cytokeratins (CK). In addition, we have characterized variations in intrinsic fluorescence, specifically at red wavelengths (650-670nm) as a potential signature for distinguishing contributor cell populations. Results from HLA hybridization experiments showed that surface antigens on cells transferred from the palmar surface onto a substrate are largely unreactive. Cells showed greater interaction with Cytokeratins probes, but we did not observe consistent differences across contributor cell populations. Flow cytometry analysis did show distinct variation in red autofluorescence profiles between some contributor cell populations, with median fluorescence intensities ranging from ~200 RFU to ~1,200 RFU. Results from controlled touch experiments suggest that variations in autofluorescence may be due in large part to the transfer of exogenously-derived fluorescent compounds to the contributors' palmar surface which are then co-deposited with intact epidermal cells onto the touch surface.

The goal of the next phase of the project was to test whether optical differences between contributor cell populations could potentially be used as the basis for a cell separation workflow prior to genetic analysis to ultimately obtain single source STR profiles without any ambiguity or complex pattern interpretation. To accomplish this, controlled two person touch mixtures were created, separated into two fractions via Fluorescence Activated Cell Sorting (FACS) using gating criteria based on intensity of 650-670nm emissions, and then subjected to standard caseworking DNA analysis techniques. Overall, STR typing of the sorted fractions from touch mixtures yielded at least partial profiles that were consistent with separation of individual contributors from the mixture. The prevalence of extracellular DNA and its subsequent loss during the sorting process likely contributed to the limited DNA recovery and detection of only partial STR profiles from these touch samples. Follow up experiments confirmed that 'cell-free'

or extracellular DNA can constitute a significant proportion of the total genomic content for many types of touch epithelial cell samples. Nevertheless, our project results demonstrate that separation of contributor cell populations based on intrinsic attributes of epidermal is possible and may potentially be used in conjunction with standard DNA caseworking protocols to decrease the complexity/ambiguity of mixed STR profiles. This research can significantly impact the forensic science community by introducing a new analytical method that can help reduce the analytical bottlenecks, inconclusive results, and loss of evidence that often accompany mixed STR profile interpretation within forensic caseworking units.

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Executive Summary

Analysis of ‘touch’ or trace epithelial cell mixtures is a significant problem for DNA caseworking units. Currently, interpretation of STR profiles containing multiple contributors requires time-consuming and frequently subjective procedures that often decrease the probative value of the evidence (sometimes completely). There remains a considerable need for front end techniques that can separate cell populations from different contributors prior to DNA analysis thereby facilitating the generation of single source STR profiles and/or simplifying multi-contributor samples. Although a number of methods exist for selectively labelling and isolating cells from a mixture, they generally have proven to be effective only for resolving mixtures with multiple cell types (e.g., blood-saliva, sperm-epithelial) and have not been tested on ‘touch’ mixtures that consist solely or largely of sloughed epidermal cells.

Therefore, the objective of this study was to characterize the optical properties and immunochemistry of cells recovered from touch or contact biological samples with the overarching goal of identifying biomolecular targets that may be used to differentiate, and ultimately separate, epidermal cell populations from different individuals. We initially focused on the reactivity of epidermal cells to antibody probes that target two different protein classes: the Human Leukocyte Antigen (HLA) complex and cytokeratins (CKs). We followed these experiments with a survey of intrinsic fluorescence of epidermal cells from different contributors at red wavelengths (650nm-670nm). Next, in an effort to physically isolate contributor cell populations in a controlled two-person ‘touch’ mixture, we used the observed inter-contributor variation in autofluorescence profiles to develop gating criteria for subsequent fluorescence activated cell sorting (FACS). We processed the sorted cell populations using standard forensic DNA analysis methods, and compared the STR profiles of each fraction against profiles from the

unsorted mixture and the contributor reference samples in order to evaluate the efficacy of separation. Lastly, our sorting results lead us to investigate the role and relative contributions of ‘cell-free’ or extracellular DNA in touch samples as this can impact the persistence of genomic material associated with intact cells through the sorting process and, ultimately the probative nature of the resulting DNA profile.

Results from our initial flow cytometry analysis of touch samples showed that biological material recovered from standard sampling swabs and eluted in solution was composed of intact cells consistent in size with corneocytes (20-40 μ m) and smaller, irregular events. Single cell imaging of the latter fraction suggests that it is composed of cellular debris, deformed/damaged cells, and fiber fragments that may originate from the collection swab or are associated with the sampled substrate. Over the course of this study we observed both inter- and intra-contributor variation in the number of corneocytes detected and their percentage relative to the total number of events in a touch sample. Nonetheless, cell yield was not an issue as touch swabs routinely provided more than 10,000 cells for analysis.

Hybridization experiments targeting HLA antigens on the cell surface showed little to no binding to either allele-specific or class-level antibody probes, suggesting that HLA antigens were either not present or were unreactive. The absence of HLA probe interactions in this study is further evidence that the overwhelming majority of cells in these touch samples are fully differentiated keratinocytes, which have been shown to display limited reactivity to HLA Class I probes in contrast to cells derived from deeper layers of the epidermis or non-epidermal epithelial cell sources.

Subsequent hybridization experiments targeted cytokeratins, which are an important structural component of both differentiating and fully differentiated epidermal cells. Specifically,

we utilized AE1 probe which binds to cytokeratin proteins 10, 14, 15, 16, and 19. We found that touch samples consistently hybridized to the AE1 probe, albeit donors displayed slight variation in binding affinity. Across sampling days, the degree of variation occasionally increased, however, we observed that the difference was sometimes minimal. These results suggest that cytokeratin expression – at least on the pan-level that is capable of being explored with a probe such as AE1 – may not present a consistently useful means of discriminating between individuals.

Surveys of red autofluorescence profiles (650-670 nm) showed greater levels of variation between contributors although the degree of differentiation (or conversely overlap) between autofluorescence profiles varied considerably across days, even between the same two individuals. Nonetheless, the median fluorescence across certain contributor pairs ranged from ~500 to 3000 RFUs. Microscopic surveys of individual cell events showed that red autofluorescence is associated with what appear to be intact corneocytes. Fluorescence was also observed associated with other flow cytometry events, which could be rolled or fragmented cells, or possibly non-cellular material such as fibers.

We then investigated the possible causes for this phenomenon. Differences in the histogram profiles in replicate samples from the same donor sampled on different days suggests that red autofluorescence may partly be driven by contact with exogenous substances prior to depositing a touch sample. To test this, we executed a series of controlled experiments whereby donors handled a series of substances with either known or suspected autofluorescent properties prior to depositing a touch sample. These included nitrile laboratory gloves, plant material, and marker ink. Results indicate that the level of red autofluorescence in touch samples can be influenced by a donor's contact with these specific materials prior to handling the substrate from

which cells were collected. The autofluorescence could be easily visualized microscopically or using flow cytometry, and persisted after hand washing. This trend was consistent across multiple donors for each of the substrates.

To test whether differences in autofluorescence could potentially be used as the basis for a cell separation workflow, controlled two person touch mixtures were separated into two fractions via Fluorescence Activated Cell Sorting (FACS) using gating criteria based on intensity of 650-670nm emissions, and then subjected to DNA analysis. Genetic analysis of the sorted fractions provided partial DNA profiles that were consistent with separation of individual contributors from the mixture suggesting that variation in autofluorescence signatures, even if driven by extrinsic factors, may nonetheless be a useful means of isolating contributors to some touch mixtures.

The consistently low DNA yield observed sorted cell fraction led us to investigate the prevalence of extracellular DNA in touch samples and its physical/structural relationship to intact epidermal cells. We found that the vast majority of DNA (84-100%) detected in these touch samples was extracellular and was uncorrelated to the number of epidermal cells detected. High resolution chemical force microscopy showed that portions of extracellular DNA were loosely associated with the cell surface and could be easily removed through water washing while other fractions were conjugated to the cell surface.

This investigative study marks an important foundation for ongoing research into methods that facilitate the separation of touch samples into individual contributor cell populations for downstream DNA analysis. While additional research is needed before flow cytometry can be imported as a front end technique in forensic DNA casework, our results indicate that there are features of fully differentiated keratinocytes, such as red autofluorescence

profiles, that can be harnessed to distinguish cell populations from some individuals. A benefit of a feature such as red autofluorescence is that it can be measured without the need for antibody probes or other special reagents, allowing for touch samples to be pre-screened for this trait. Further, intrinsic fluorescence profiles can be integrated with other cell sorting platforms currently being investigated/developed.

Introduction

A. Statement of the Problem

Analysis of biological mixtures has been an issue in the forensic community since the introduction of molecular methods for human identification. The presence of cells (and, therefore, DNA) from multiple individuals in a biologic stain makes DNA profiles difficult or even impossible to interpret, often leading to loss of evidence. Mixture interpretation protocols are technically challenging and the community suffers from a lack of standardized interpretation procedures. Although probabilistic genotyping systems can perform analyses on complex mixtures which are superior to human analysis, implementation of these systems poses a number of challenges (e.g. cost; time requirements; legal skirmishes over proprietary software; difficulties associated with communicating probabilistic information to a jury), mis-estimation of the number of contributors to a sample can affect probabilistic results and there are limits as to the number of contributors that can be successfully disentangled.

Selective labelling of surface antigens coupled to cell separation techniques has emerged as a promising approach for isolating cells from different contributors prior to DNA extraction. The non-destructive nature of cell labelling and high throughput capabilities of fluorescence-based cell sorting can be easily integrated within the operational workflow of forensic casework,

and provide a powerful front-end technique to generate single source STR profiles from complex mixtures. However, these methods have only been demonstrated on a limited number of tissue types (e.g., blood, vaginal, buccal cells) and have not been explicitly tested on ‘touch’ biological samples which are composed almost entirely of sloughed epidermal cells which have vastly different biological and structural properties from other forensically relevant cell types. There are a number of molecular tools and methodological strategies that may facilitate the recovery and sorting of individual contributors’ cells, these methods have rarely been tested in a forensic context and therefore, have unexplored potential for isolating cell populations from mixtures and enabling single-source STR profile analysis.

B. Literature Citations/Review

DNA mixtures are a ubiquitous problem for forensic laboratories. Considerable effort has been made to establish best practices for interpreting DNA profiles containing multiple contributors (1) and several novel statistical approaches for mixture interpretation have also been reported (2–6). In addition to new guidelines and numerical methods, many laboratories have developed methods to help separate different components of a biological mixture prior to DNA extraction. Most of these techniques have focused almost exclusively on mixtures containing sperm cells and epithelial cells such as differential lysis (7), centrifugation (8,9), and Laser Capture Microdissection (LCM) (10). While some of these procedures have been shown to increase the resolution of individual components in a sperm-epithelial mixture, most are limited by high sample requirement or result in incomplete separation and low cell yields (10). A variety of microfluidic platforms have also been developed for extracting single-source DNA profiles from biological mixtures and have proven to be effective techniques for separating sperm from

epithelial cells prior to DNA extraction (11,12) but have not been demonstrated on other types of forensic mixtures (e.g., touch or trace cell mixtures).

Recently, there has been extensive work with High Throughput Sequencing (HTS) and its applications for resolving complex biological mixtures (13,14). The technology offers many potential benefits for forensic laboratories including the high volume of sequence data and the ability to process degraded samples. However, the data interpretation requires integrated bioinformatics algorithms and allele assignments are still intrinsically probabilistic. Further, many of the HTS platforms have a sequencing error rate incompatible with forensic casework (15). Because of these factors there is still a considerable need for physical separation techniques on the front end of forensic DNA workflow that facilitate the generation of single source STR profiles from biological mixtures.

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Antibody Labelling and Cell Sorting

Cellular immunohistochemistry offers one promising avenue for individualization of cell mixtures; for example, exploiting proteins within the Human Leukocyte Antigen (HLA) Class I complex. HLA molecules are surface expressed glycoproteins that play a major role in immune system function. Most antigens initiate cytotoxic T-cell responses or antibody production from B-cells when presented with foreign peptides. HLA proteins are expressed on nearly every type of nucleated cell.

The most important feature of HLA antigens for the purpose of mixture separation is their diversity. The group of genes comprising the HLA loci is one of the most polymorphic coding regions in the human genome (16) and each HLA subgroup has several hundred possible alleles. Most of the allelic variation results in structural changes to the surface-expressed protein. The

frequency of each HLA allele has been documented in worldwide databases (17) and range from extremely common (possessed by ~45% of the population) to relatively rare (possessed by <1%).

HLA antigens are discriminating, though not so much so as STR profiles; this is precisely what makes them so well-suited for mixture separation. In order to sort an unknown mixture into its individual components, markers are needed that are likely to be shared by some contributors to a mixture and not others, and which can be combined into a set to be made increasingly more discriminating. The population frequency of most HLA alleles gives these molecules enormous potential for differentiating cells within a complex mixture. Overall, the population frequency of individual HLA-A, -B, and -C alleles ranges from <1% to ~45% and varies across ethnic groups, with a majority having frequencies less than 25% (17). For example, HLA-A*02 has the highest incidence in Caucasians (~45%) and significant lower frequency in Hispanic, African-American, and Asian groups (~24% and 20%, and 18%, respectively). Including a probe that targets one of the more common antigens makes it highly likely that the cells of at least one contributor to the mixture will be labelled. Combining multiple HLA probes into a single assay makes it increasingly likely that the cells of multiple—and potentially all—contributors to a hypothetical mixture will be uniquely labelled, and thus distinguished from one another.

To facilitate isolation of an individual cell population from a mixture, antibody labelling is typically coupled to a robust detection system and high throughput separation technologies. One of the more common techniques is Fluorescence Activated Cell Sorting (FACS). During FACS, each cell in a sample is passed single file in a fluid stream through a light beam. Light is scattered by the cell dependent upon its morphological characteristics (*i.e.* size, granularity), and light of specific wavelengths interacts with antibody-coupled fluorophores bound to the cell

surface, producing fluorescence of an intensity proportional to the number of fluorophores present. Current FACS instruments have the capability to individually detect and quantify the light scattering and fluorescent characteristics of thousands of cells per second (18,19). FACS also allows for physical isolation of cells that satisfy a certain set of criteria or 'gate'. Gating involves setting upper and lower limits for one or more parameters of interest, such as fluorescence intensity. At the same time that sensors are collecting a cell's light scatter and/or fluorescent characteristics, a computer sensor determines whether those characteristics satisfy a defined gate. If they do, the cell is diverted toward one container through the use of electromagnetic deflector plates; if they do not, the cell is diverted toward another container. In practice, this means that cells bound to fluorescently-labeled antibodies can be separated from unconjugated cells within a mixture.

FACS has several advantages for forensic casework. First, FACS instruments can process thousands of cells per second and sort based on morphology, composition, or antibody tagging (19). Second, cell sorting is an inherently non-destructive technique that allows for low numbers of target cells to be analyzed and isolated without sample loss (20). Third, flow cytometry-based cell separation methods are extensively published and these procedures are routinely conducted at most major hospitals and research facilities. Additionally, the physical dynamics of droplet formation and the sensitivity of optical detection systems make FACS a more robust technique for cell separation than other types of antibody labelling and cell enrichment systems such as column capture and/or micro-bead based binding systems which can be more prone to cross-contamination of cell populations (21).

FACS-based Separation of Touch Mixtures

Because of these factors there has been considerable interest in FACS-based approaches for resolving complex cell mixtures, in particular forensic samples involving pooled blood or blood mixed with cells from other tissues. The earliest studies focused on resolution of sperm and epithelial cell mixtures and employed fluorescently labelled antigens to selectively label and then isolate sperm cells (22). Other efforts have applied tissue-specific antibody labelling and FACS to isolate blood cells from a mixture with buccal cells (23). Most recently, this approach has been used to isolate cells from one individual in complex blood mixtures containing as many as four contributors and demonstrated that unambiguous single source STR profiles could be obtained from mixtures containing only one cell type (24).

Despite these successes, the primary limitation of previous FACS-based studies is that they have focused primarily mixture samples that that not include touch or trace epidermal cells. Specifically, sloughed epidermal cells (aka corneocytes) come from the outermost layer of the skin, the stratum corneum. Prior to shedding, corneocytes have undergone a process of terminal differentiation as they migrate to the outer layer whereby they have lost their cellular organelles including their nuclei (25,26). Additionally, various keratin molecules accumulate both within and upon the surface of the cell as it becomes encased within an extracellular matrix composed of lipids and hydrophobic proteins that contribute to the skin's barrier function (27,28). The progressive keratinization of epidermal cells and degradation of their intracellular components may pose considerable obstacles to the development of immunochemical techniques to differentiate contributors in touch mixture samples. Surface antigens, which can be targeted for selective labelling of individual cell populations in a forensic mixture (23,24,29), have variable reactivity in keratinocytes originating from different epidermal layers (e.g., basal layer is more

reactive than spinous layer) (30–32), and their utility has yet to be explicitly evaluated in touch samples.

C. Statement of Hypothesis/Rationale for Research

Therefore, the objective of this study was to characterize the optical properties and immunochemistry of cells recovered from touch or contact biological samples and identify biomolecular targets that may be used to differentiate, and ultimately separate, epidermal cell populations from different individuals using Fluorescence Activated Cell Sorting prior to DNA analysis. By separating whole cells from a forensic mixture prior to DNA extraction and amplification, single source STR profiles can be generated without any ambiguity or complex pattern interpretation.

This aims and scope of this project specifically address two operational requirements identified by the 2014 Forensic Technology Working Group:

- Ability to differentiate, physically separate, and selectively analyze DNA and/or cells from multiple donors or multiple tissue/cell types contributing to mixtures
- Ability to differentiate and tag a cell, identify and associate the biological source through to profile generation

II. Methods

Collection of touch samples for antibody hybridization, imaging flow cytometry, and conventional flow cytometry

Touch samples were obtained pursuant to VCU-IRB approved protocol ID# HM20000454_CR3. Volunteers were asked to rub a sterile polypropylene conical tube (P/N

229421; Celltreat Scientific) using their palm and fingers for five minutes. Cells were collected from the surface with sterile pre-wetted swabs (P/N 22037924; Fisher Scientific) followed by dry swabs. A total of six wet swabs and two dry swabs were used to sample the entire tube surface. To elute the cells into solution, the swabs were manually stirred then vortexed for 15 seconds in 10 mL of ultrapure water (18.2 M Ω ·cm). The entire solution was then passed through a 100 μ m filter mesh prior to antibody hybridization, conventional flow cytometry, and imaging flow cytometry (IFC). Separate aliquots of the resulting cell solution were used for each analysis method.

Collection of touch samples after handling specific materials

For studies of exogenous influences on autofluorescence in touch samples, each donor handled a specific material prior to depositing cells on a conical tube. These materials included purple nitrile gloves (Precision® brand, powder-free, P/N PCS775), plant material, and conical tubes marked with Sharpie® marker ink. Prior to handling these materials, donors washed their hands with antibacterial soap under running water for 15 seconds and then allowed them to air dry. For nitrile glove experiments, donors wore a nitrile glove on their right hand, leaving the left (control) hand bare, and proceeded to grip/handle various items with their gloved hand for five minutes to simulate normal activity (e.g. pipette, door handle, tools). The glove was then removed and the contributor held a conical tube in each hand for five minutes. For experiments involving plant material, subjects handled individual leaves of kale or collard greens using only their right hand for five minutes (left hand was not used and served as a control cell population). The handling procedure involved lifting/tossing leaves with fingers and palmar surface and

tearing individual leaves into smaller pieces, approximating how this material might be handled during food preparation. Subsequently, donors rinsed their hands with water for approximately five seconds (to remove pieces of plant material) and allowed their hands to air dry before depositing touch samples by holding a conical tube in each hand for five minutes. For marker ink experiments, each donor held a conical tube that had been marked with a black or green marker in his/her right hand for five minutes before depositing touch samples by holding an unmarked conical tube in each hand for five minutes. For each of these experiments, cells were collected from the surface of each tube and eluted into solution as described above. Separate aliquots of the resultant cell solutions were used for flow cytometry analysis and IFC.

Collection of touch samples after handling material and hand washing

To test the effect that hand washing has on exogenous sources of autofluorescence in contributor cell populations, donors were asked to manipulate kale leaves with both hands (lifting, tossing and tearing, as described above). Each donor then held a wooden-handled kitchen knife with one hand for five minutes. Next, the donors washed both hands with soap and water for 15-20 seconds and allowed their hands to air dry. Finally, each donor held a second knife in his or her other hand (i.e., the hand that was not used to hold the first knife) for five minutes. Cells were collected from the handle of each kitchen knife as described above. Cell solutions derived from the unwashed hand were compared to cells solutions from the hand that had been washed immediately prior to deposition using flow cytometry as well as IFC. Because both hands were used to handle plant material during this experiment, negative control cell populations from the same donor (where cells were deposited without any immediate prior contact with plant material) were collected on a separate day and analyzed using flow cytometry.

Collection of touch samples for mixture studies

To test whether observed variations in autofluorescence between two touch sample contributors could be used to successfully separate a mixture of their cells, each donor rubbed a conical tube as described above. The surface of each donor's tube was swabbed with one slightly wetted cotton-tipped swab followed by one dry swab. The swabs were then eluted in 2mL of sterile water, vortexed for 15 seconds, and passed through a 100 μ m mesh filter. An 860 μ L aliquot of each donor's touch cell solution was combined to create a 1:1 mixture (by vol.) for flow cytometry analysis, gating, and subsequent sorting via FACS. Another 200 μ L from each donor was combined to create a mixture that would proceed directly to DNA analysis without sorting (i.e. to develop an unsorted mixture profile for comparison). The remaining cell solution for each of the two donors was utilized for IFC studies.

Antibody Hybridization

Three milliliter aliquots of donors' touch cell solutions were centrifuged at 5,000xg for five minutes. The resulting cell pellets were then dissolved in \sim 100 μ L of supernatant and incubated for 10 minutes with 1 μ L of Human Fc Receptor block (Cat# 130-059-901, Miltenyi Biotec) to increase the specificity of antibody binding before reaction with either HLA or CK probes. For HLA hybridizations, cells were incubated with mouse anti-human monoclonal antibody (mAb) HLA-ABC-FITC (Cat# 311403, BioLegend) for 30 minutes. Cells incubated with anti-mouse IgG2a-FITC (Cat# 343303, BioLegend) for 30 minutes served as the isotype control for these experiments. Cells were then washed once in 1x FACS buffer [PBS supplemented with 2% Fetal Bovine Serum (FBS, Cat# 100-106, Gemini BioProducts) and 10%

Sodium Azide (Cat# S2002, Sigma-Aldrich)] and re-suspended in the same solution until flow cytometry analysis.

For CK hybridization experiments, cells were incubated with anti-acidic cytokeratin probe ('AE1' (recognizes CKs 10, 14, 15, 16 and 19), Cat# 14-9001-80, Affymetrix eBioscience) for 30 minutes followed by reaction with a secondary antibody, anti-mouse IgG1-APC (Cat# 17-4015-80, Affymetrix eBioscience). We used anti-mouse IgG1-APC (Cat#17-4714-42, Affymetrix eBioscience) to create the isotype control for AE1 experiments, incubating for 30 minutes. As before, cells were washed once and then resuspended in 1x FACS buffer prior to analysis.

Imaging Flow Cytometry

For fluorescence imaging, intact epidermal cells were first isolated from ~500 μ L aliquots of touch sample cell solutions by sorting the "large cell" fraction (i.e., 'K' subpopulation in FSC-SSC plots described in (33)) into a collection tube using a BD FACSAria™ II (Becton Dickinson) flow cytometer with 488 nm and 633 nm coherent solid state lasers, and set to the following channel voltages: FSC,200V; SSC,475V. The sorted cell solution (containing at least 1,000 events) was then analyzed using an Amnis® Imagestream X Mark II (EMD Millipore) equipped with 488nm and 642nm lasers. Images of individual events were captured in the Brightfield channel and APC channel (642-745nm). Magnification and focus settings varied with cell size. Cell images were analyzed and exported with the IDEAS® Software (EMD Millipore).

Flow Cytometry and Fluorescence Activated Cell Sorting

For HLA and CK studies, flow cytometry analysis was performed on the BD FACSCanto™ II Analyzer (Becton Dickinson) equipped with 488nm and 633nm lasers. Channel voltages were set as follows: Forward Scatter (FSC, 150V), Side Scatter (SSC, 200V), Alexa Fluor 488 (FITC, 335V), Phycoerythrin (PE, 233V; PE-Cy5, 300V; PE-Cy7, 400V), and Allophycocyanin (APC, 250V). For each experiment, 10,000 total events were collected for analysis. Data analysis was performed using FCS Express 4 Flow Research Edition (De Novo Software).

Intrinsic fluorescence studies of touch samples and Fluorescence-Activated Cell Sorting (FACS) of two-person epidermal cell mixtures were performed on one of two BD FACSAria™ II (Becton Dickinson) flow cytometers, each employing 488 nm and 633 nm coherent solid state lasers. On each instrument, channel voltages were set as follows: FSC, 200V; SSC, 475V; APC, 400V. Events falling into the “large cell” gate were analyzed for red autofluorescence (650-670nm), again using FCS Express 4 Flow Research Edition. Comparisons between fluorescence intensity histograms were generally made for the distribution of events fluorescing between 1 and 10⁴ RFUs. For mixture samples, sorting gates were set to enrich for each of the two contributors in the mixture based on their individual autofluorescence profiles (‘P9’ and ‘P10’ regions of the fluorescence histograms shown in Figure 6). The majority of the cell solution aliquot (1,720 μ L) was processed through FACS, with a small amount left unprocessed to prevent introduction of air bubbles.

DNA Extraction, Purification and Quantitation

Sorted samples were centrifuged at 10,000 \times g for 15-20 minutes to pellet cells. The supernatant was concentrated onto a YM-100 Microcon filter (P/N 42413, EMD Millipore) and

eluted in 25 µl of sterile distilled water, then re-combined with the cell pellet. These samples, as well as the unsorted mixture sample and reference samples (donor buccal samples) were each lysed and purified using the DNA IQ System (Cat# DC6701, Promega) following the Virginia Department of Forensic Science (VA-DFS) standard protocols (Virginia Department of Forensic Science 2015). DNA extracts were quantitated using the Plexor HY System kit (Cat# DC1001, Promega) coupled with the Stratagene MX3005P Quantitative PCR Instrument and Plexor Analysis Software.

STR Amplification and Profiling

We used the PowerPlex® Fusion System kit (Cat# DC2402, Promega) to amplify STRs in an ABI 9700 thermal cycler, following the manufacturer's protocols. Capillary electrophoresis was performed on the ABI 3500 xL Genetic Analyzer (Life Technologies) as described in the instruction manual, and resulting data was analyzed using GeneMapper ID® X v1.4 Software (Life Technologies) according to the manufacturer's recommendations. The analytical thresholds used to interpret the resulting data were dye-specific and set at 88 relative fluorescent units (RFU) for fluorescein, 74 for JOE, 114 for the TMR-ET, and 80 for CXR-ET. The stochastic threshold was set at 396 RFU.

Cell Enumeration

In order to precisely quantify the cells in our samples during certain flow cytometry experiments, we spiked our cell solutions with a known concentration of 123 eBeads (01-1234-42; Affymetrix eBioscience), fluorescently-labeled microparticle standards that are 7 µm and easily distinguishable from our target cell population both in size and fluorescence (FITC, PE,

and APC channels). The ratio of cells to beads was then used to determine the concentration of cells following the manufacturer's protocol.

III. Results

A. Statement of Results

Morphological Characterizations

Initial optical characterizations of cell solutions recovered from touch samples showed two distinct populations. Individual events within the 'K' gate measured ~20-40 μm in diameter. The size and morphology of the subset of large cell (K) fraction events imaged with AMNIS (typically several hundred cells per sample) appeared to be consistent with intact keratinocytes (top two rows of cell images in Figure 1); we did not observe any cells with features that would suggest the presence of other epithelial cell types (e.g., buccal). Evidence of folded or rolled cells was also observed in the K population which likely reflects physical deformation of some cells during surface swabbing. Events within the 'D' population were typically less than 10 μm . Their size and overall variable morphology in AMNIS images (bottom two rows in Figure 1) suggest that these events represent cell fragments, biological debris, or non-cellular particles such as fibers. The distribution of cell events within the K-population vs. D-population were observed to show considerable variation both between donors and between sample replicates from the same individual.

Antibody Labeling Experiments

Touch samples from ten donors were each hybridized to a fluorescently-labelled pan-HLA antibody that recognized all antigens within the A, B, and C protein classes. Probe-hybridized cells displayed no increase in average fluorescence when compared to unlabeled cells or isotype controls (Figures 2a-c). Similar results were obtained when HLA probes specific for the A*02 allele were hybridized against cells that screened positive for the A*02 allele (data not shown). Although there was no discernable change in fluorescence after probe hybridization, some differences in the distribution of FITC channel intrinsic fluorescence values were consistently observed from one donor to the next (e.g., compare purple and dark blue histograms in Figures 2a-c).

In contrast, experiments using AE1 cyokeratin antibodies show probe uptake for each of ten donor samples tested when compared against unstained cells and isotype controls (compare Fig. 2f to Figs. 2d and 2e). We observed slight inter-individual variation in binding efficiency. Mean fluorescence intensities ranged between 417 and 663 relative fluorescence units (RFUs), with all donors exhibiting significant overlap in their histogram profiles. Of note, one donor cell population showed higher average levels of intrinsic APC channel fluorescence compared to other donors (maroon histogram in Figs. 2d-e). Interestingly, this donor cell population displayed one of the lowest probe binding efficiencies of those surveyed (maroon histogram in Fig. 2f). When touch samples from a subset of these donors were monitored for changes in AE1 uptake between sampling days, we found that the efficiency of probe binding varied from day to day, with samples from one donor in particular exhibiting discernibly higher fluorescence than other donors on two of the four collection days (Figure 3, red histograms).

Intrinsic Fluorescence Surveys

Next, we examined variation in intrinsic fluorescence at red wavelengths (~650-670 nm) as a potentially discriminating characteristic for cell populations from different individuals. This wavelength was chosen based on initial observations in the course of antibody hybridization studies that unstained cell samples from some contributors showed higher mean fluorescence intensities than others (Figs. 2d and 2e (maroon histograms)).

Fluorescence histograms of individual cell populations from eight different donors are shown in Figure 4. For ease of comparison and visualization, profiles have been overlaid and grouped by the day on which cells were deposited, collected, and analyzed by flow cytometry. Clear differences in the red fluorescence (APC) channel are observed between several pairs of donor cell populations, particularly J16-D02 during the first experiment and J16-S07 in the second experiment (Figures 4a and 4b respectively; Table 3). Most experiments resulted in one or more contributor cell population(s) whose fluorescence profile(s) could be distinguished from the others collected that day, such that a fluorescence intensity gate could be designed that would be expected to capture that contributor's cells to the exclusion of (or minimal contribution of) cells from other contributors. However, significant and/or complete overlap was observed between many donor pairs (e.g., A42-B17 in Figure 1a; I66-S07 in Figure 4d). Sometimes, overlap of fluorescence distributions was such that gating could potentially separate the contributors into two or more groups (e.g. Fig. 4d: A42, B17, I66, R12 and S07 in one group; D02 and J16 in another group). All contributors from the final experiment exhibited overlapping fluorescence histograms (Figure 4e).

Cell populations from J16 and D02 showed a great deal of disparity in fluorescence intensity in the first experiment, such that overlap between these populations was minimal (Figure 4a). There was somewhat less distinction – and thus more overlap – observed between

the same contributors during a second replicate (Figure 4c); during a third, overlap between the two populations was substantial (Figure 4d). As these results suggest, fluorescence intensity values for cell populations derived from any given contributor varied in distribution across replicate experiments on different days. Figure 1f shows overlaid histograms for J16 cell populations; mean fluorescence intensity values ranged from 589 to 2606 relative fluorescence units (RFUs) across five sampling days (Table 3).

To test the reproducibility of intrinsic fluorescence profiles (APC channel) additional donors' touch samples were analyzed. Different subsets of these individuals sampled and analyzed on three different days; results are shown overlaid and grouped by sampling day in Figure 4. Significant overlap was observed between many of the donors on each sampling day. However, touch samples from one contributor, E15 (red histogram in each panel), consistently contained a number of cells with higher fluorescence intensity than cells from other contributors. Microscopic surveys of individual cell events from contributor E15 showed red autofluorescence associated with what appear to be intact corneocytes (Figure 7). Fluorescence was also observed associated with other flow cytometry events, which could be rolled or fragmented cells, or possibly non-cellular material such as fibers.

To investigate the consistency of autofluorescence signatures in the two most disparate individual cell populations, touch samples were collected from donors E15 and D02 on seven additional days and analyzed for red autofluorescence. Results showed that the degree of differentiation (or conversely overlap) between autofluorescence profiles varied considerably across days (Figure 6). Nonetheless, the mean APC channel fluorescence of E15 cell populations was consistently higher than D02 populations.

The observation that red autofluorescence varied between donors led us to explore the possible causes for this phenomenon. Due to differences observed in the histograms developed from touch samples collected from the same donor on different days, we hypothesized that contact with exogenous substances prior to depositing a touch sample might play a role in the observed variation. We investigated this by executing a series of controlled experiments where donors handled specific materials encountered in the laboratory (nitrile gloves, marker ink) or at home (plant material) immediately prior to depositing a touch sample.

We observed shifts in red autofluorescence of touch cell samples subsequent to handling each of the tested materials, with the degree of shift depending on the material handled. Histograms of touch samples collected from a donor after handling an item bearing marker ink on two different days (black ink one day, green ink the other) displayed shifted red fluorescence intensity histograms compared to cell populations from the control (i.e. non-marker) hand (Figure 10). While overlap was observed in the fluorescence intensities of a subset of cells from the marker and control touch samples, fluorescence-based sorting gates can be conceived that should capture a significant number of events from one cell population to the exclusion or near-exclusion of the other (e.g. one fraction < 100 RFU, second fraction > 1000 RFU).

Histograms of touch cell samples collected from donors who wore a purple nitrile glove on one hand also displayed slight shifts in mean fluorescence intensity compared to cell populations from the control (bare) hand, although considerable overlap from the two cell populations was observed (Figure 9a-c). For samples collected from a single donor on different days, fluorescence distributions of test and control cell populations varied (Figure 9c vs. 9d). The high degree of overlap observed in Fig. 9d appears to be attributable to the combined effects of lower intensity fluorescence of gloved hand (note the subset of cells fluorescing below 100 RFU,

compared with Fig. 9c) and the relatively low cell yield from the ungloved hand. This further highlights the interplay between cell yield and fluorescence distributions underlying the ability to successfully sort (and type) such samples.

We observed much more distinct shifts in red autofluorescence intensity after donors handled plant material (kale or collard greens) relative to control cell populations derived from hands that had not handled plant material (Fig. 8a-d). A subset of cells from the plant-holding hand displayed lower levels of fluorescence, on par with control cell populations; this trend was observed in samples from four different donors, although the number of cells in the subset fluorescing at lower intensity varied across donors (*e.g.*, compare Figure 8a with Figure 8c). This is likely attributable to the lack of precise control over how study participants handled plant material, which would be expected to result in a variable amount of transfer of plant material to the palms. Notably, one donor's control touch sample showed a higher mean fluorescence compared not only to other donors' control samples, but also to the low-fluorescence subset of cells from that donor's test (*i.e.* kale-handling) hand (Figure 8a). One potential explanation is the control hand made contact with some unknown exogenous fluorescent material prior to this experiment, which persisted through initial hand washing. This underscores the difficulty of controlling for all conditions that could influence a touch sample (*e.g.* Fig. 8b (control hand in nitrile glove experiment displays a secondary fluorescence "peak" around 1000 RFU suggesting a subset of palmar cells potentially associated with unknown exogenous fluorescent material)). Another possibility is an endogenous influence, though the difference observed between the low-fluorescence cells from the two hands undercuts a biological basis, which would generally be expected to affect both hands similarly.

Introducing a hand washing step subsequent to handling plant material allowed us to examine the persistence of transferred fluorescence in touch samples. A distinct rightward shift in mean fluorescence comparable to that seen in cells derived from unwashed hands was observed to persist in touch samples collected after donors washed their hands with soap and water for 15-20 seconds (compare red histograms in Figure 8e (pre-wash)) and Figure 8f (post-wash)). However, a subset of post-wash cells were observed to fluoresce at the lower intensities characteristic of control (non-plant-handling) touch samples; this suggests that washing removed fluorescent substances from a portion of the cells in the sample.

Microscopic surveys of individual events derived from the cell population represented in the red histogram from Figure 8e (Donor I66) shows that the fluorescence is associated with whole, intact cells consistent in size and morphology with keratinocytes (Figure 11), and to a lesser extent with other material in the sample that may be rolled cells, cellular debris or even non-cellular material. Similar fluorescence configurations (*i.e.* fluorescence primarily associated with apparent cell surfaces) were observed in microscopic surveys of donor E15's cell samples from our initial touch studies, where there was no deliberate touching of particular materials, and the source of fluorescence is unknown (Figure 7).

Two-person mixture study

Mixtures of cells deposited by donors D02 and E15 were sorted into separate fractions via FACS according to the gating criteria shown in Fig. 12, and then subjected to DNA analysis. “Sort A” and “Sort B” – the cell fractions that met gating criteria derived from intrinsic fluorescence measurements of cells from donors D02 and E15 (respectively) – each produced a

partial profile (Table 1). The high degree of dropout and possible drop-in alleles observed are consistent with the extremely low level of template DNA detected in each cell fraction (<50pg).

All alleles detected in Sort A were consistent with donor D02 with the exception of a single 24 allele at locus D2S1338, which did not originate from donor E15 and is likely a drop-in allele; none of E15's obligate (i.e. unique) alleles were detected in the DNA profile developed from Sort A. Likewise, all alleles detected in Sort B were consistent with donor E15 with the exception of a single 13 allele at locus D13S317, which did not originate from donor D02 and is likely a drop-in allele; none of D02's obligate alleles were detected in the DNA profile developed from Sort B.

Quantification of Epidermal Cells and Relationship to Extracellular DNA in Touch Samples

Cell counts and DNA yields were compared across 31 touch samples generated from eight different individuals that used both dominant and non-dominant hands to hold the substrate. To investigate the effect of hand washing on the transfer of cellular and extracellular components of a touch sample, half of these samples were collected after donors had washed their hands and the other half without immediate hand washing.

An estimated $\sim 5 \times 10^3$ to $\sim 1 \times 10^5$ cells were recovered from washed hand samples, versus $\sim 1 \times 10^3$ to $\sim 8 \times 10^4$ cells from unwashed hand samples (Figure 14). Overall, we observed greater transfer of cells in the washed hand samples than the unwashed hand samples (median of 2.5×10^4 cells vs. 8.6×10^3 cells, respectively). There was no apparent correlation between cell yield and the use of dominant or non-dominant hand when holding the substrate.

Despite the often high recovery of cells from touch samples, DNA recovery from the cell pellet was consistently low, whether from washed or unwashed hands. DNA was detected in the

cell pellet of one unwashed hand sample (0.220 ng) and three washed hands samples (0.049, 0.042, 0.060 ng). No DNA was detected in any of the other cell pellets.

In contrast, consistent differences were observed in eDNA recovery from samples generated from washed versus unwashed hands. Little to no DNA was recovered from the extracellular fraction of touch samples left by donors who had washed their hands, with quantitation values ranging from zero to 0.242 ng (Fig. 14c). In samples from unwashed hands, extracellular DNA recovery varied between zero and 4.646 ng (Fig. 14a). There was no apparent correlation between the number of cells and the quantity of DNA recovered from the samples (either eDNA or cell pellet). Neither could DNA recovery with or without hand washing be correlated to hand dominance, in contrast to findings by others (34).

The additional 19 samples tested for relative quantity of eDNA versus intracellular DNA produced results that are consistent with the above findings (Table 2, compilation of all samples (n = 35) without hand washing). In samples where DNA was detected, the total proportion of eDNA ranged from 84-100% with the majority of the samples at or near 100%.

Single cell analysis of extracellular DNA on epithelial cell surfaces

To observe the spatial distribution and physical relationship of extracellular DNA ('eDNA') to cell surfaces, recognition force mapping on both sloughed epidermal and buccal cells were conducted in a liquid microenvironment using functionalized cantilevers with specificity for cell-surface DNA. Using biomolecule modified AFM tips as probes, interaction forces between tip-bound ligands and cognate surface-bound receptors (or vice-versa) can be measured. Cells from three different individuals were analyzed with AFM force mapping using a lactoferrin probe. At points where the probe encounters surface eDNA, a higher binding force is

observed resulting in an “interaction” event.

Figure 15 shows the scan of a small area (5 μm x 5 μm) of each kind of cell (buccal and sloughed epidermal). As observed in this image, the panels b and d show the interaction forces where the white to dark dots represent the higher likelihood of a binding between the lactoferrin and surface DNA. Interestingly, both the keratinized palm and non-keratinized buccal cells show the presence of *e*DNA. Based on the mapping, the *e*DNA levels can be quantitatively analyzed. Here, the binding %, which is calculated as the ratio of the number of points showing binding events to the total number of points collected on the cell surface, can present the relative cell surface DNA content. It is important to note that in estimating the binding %, smaller areas (e.g. 5 μm x 5 μm) studied on the cell surface provide the same value as observing the entire cell (e.g. 25 μm x 25 μm). In these experiments, the cells were imaged before and after washing to observe all DNA on the surface, including both bound and loosely bound DNA. Extracellular DNA can include both membrane-bound DNA as well as any other DNA from exogenous sources including secondary sources (such as those obtained by contact). The cells were imaged again following 3 washes that removed most of the loosely bound DNA. At least 3 cells from each donor were imaged. As part of this study, we studied the quantitative monitoring/detection of the *removal* of the extracellular DNA through water washing steps. This is important in understanding the recovery and analysis of DNA obtained from forensic samples that may undergo processing in the laboratory setting. Results showed that the average DNA content of both buccal and contact cells decreased after three washes. For buccal cells, unwashed surfaces ranged between ~9% and 16% and washed surfaces ranged between ~5% and 6%. For contact (palm) cells, unwashed cells ranged between 4% and 10% whereas washed cells ranged between ~1% and 3% (The aggregate data after washing is presented in Figure 16). Interestingly, a

distinct variation in average DNA content was observed between buccal and contact cells after water washing (~6% for buccal, and ~2% for contact). In comparing with the data on unwashed cells, it appears that contact cells may contain less surface-bound DNA. This is consistent with preliminary bulk-level studies of extracellular DNA from contact and buccal cells (Figure 17) as well as previously published data from our group and others. Finally, to confirm the specificity of lactoferrin to cell surface DNA, a control experiment was conducted by adding DNase solution into the cellular micro-environment. The same area was monitored in real time on cells before and after incubation with DNase, which is expected to chop the DNA on the cell surface. Thus, a change in surface expression would be indicative of the membrane attached DNA. Expectedly, the cell surface DNAs decreased significantly after the DNase incubation (Figure 16), indicating that the detection of DNAs on untreated cells is from specific interactions between the DNA molecules and the lactoferrin probe. Because AFM is an inherently non-destructive technique, surface DNA signatures can potentially be used as a pre-screening technique to determine cell type and theoretical DNA yield from biological evidence.

B. Tables

Table 1. Powerplex fusion profiles developed from donors D02 and E15 reference samples

| | | | | | | | | |
|-----------------|-----------------|----------------|---------------------|-----------------|----------------|----------------|--------------------|---------------|
| | D3S1358 | D1S1656 | D2S441 | D10S1248 | D13S317 | Penta E | D16S539 | D18S51 |
| D02 Ref | 15 | 12,17.3 | 14,15 | 15,16 | 11,14 | 10,18 | 11,13 | 13,14 |
| E15 Ref | 15,17 | 13,16.3 | 10,14 | 13,14 | 9,11 | 16,21 | 10,12 | 13,18 |
| Pre-sort | 15,17 | 12,13, 16.3 | 11.3,14 | - | - | - | 10,11,12,13, 14 | 13,18,20 |
| Sort A | 15 | 12 | - | - | - | - | 13 | 13 |
| Sort B | 15,17 | - | - | - | 13 | - | 10 | - |
| | D2S1338 | CSF1PO | Penta D | TH01 | vWA | D21S11 | D7S820 | D5S818 |
| D02 Ref | 18,20 | 11,12 | 11,15 | 9 | 18,19 | 28,33.2 | 8,12 | 12,13 |
| E15 Ref | 19,25 | 10,11 | 11,13 | 7 | 16,17 | 30,31.2 | 12 | 11,12 |
| Pre-sort | 17,18,20, 25 | 10 | - | 7,9 | 17,18 | - | 12 | - |
| Sort A | 24 | - | - | - | - | - | - | - |
| Sort B | 25 | - | - | 7 | 16 | - | - | - |
| | TPOX | DYS391 | D8S117 9 | D12S391 | D19S433 | FGA | D22S1045 | |
| D02 Ref | 8 | - | 12,13 | 19,21 | 14,15 | 24,25 | 15,16 | |
| E15 Ref | 8,12 | 11 | 14,16 | 18,20 | 14,16.2 | 25,27 | 15 | |
| Pre-sort | 8 | - | 8,12,13, 14,16 | 18,19,20,2 1 | 14,15 | 23,25,27 | 16 | |
| Sort A | - | - | 12,13 | 19 | 14,15 | - | - | |
| Sort B | - | - | - | - | 14 | - | - | |

Table 2. Proportion of DNA in Supernatant and in Cell Pellet after Three Washes

| Sample | Extracellular DNA (ng) | DNA in Cell Pellet (ng) | Percentage eDNA |
|------------|------------------------|-------------------------|-----------------|
| D11 | 0.607 | ND | 100 |
| | 1.191 | 0.068 | 95 |
| | 0.603 | ND | 100 |
| | 0.842 | ND | 100 |
| | 2.023 | ND | 100 |
| E14 | 2.296 | 0.037 | 98 |
| | 1.134 | 0.220 | 84 |
| | 0.504 | ND | 100 |
| | ND | ND | n/a |
| E15 | 2.162 | 0.284 | 88 |
| | 0.567 | ND | 100 |
| | 4.646 | ND | 100 |
| | 0.940 | ND | 100 |
| | 2.842 | ND | 100 |
| C81 | ND | ND | n/a |
| | ND | ND | n/a |
| | 0.282 | ND | 100 |
| D02 | ND | ND | n/a |
| | 0.374 | ND | 100 |
| | ND | ND | n/a |
| | 0.028 | ND | 100 |
| | ND | ND | n/a |
| H73 | ND | ND | n/a |
| | ND | ND | n/a |
| | 0.780 | ND | 100 |
| I66 | 0.286 | ND | 100 |
| | 1.240 | ND | 100 |
| | 1.110 | ND | 100 |
| J16 | 1.804 | 0.021 | 99 |
| | 1.262 | ND | 100 |
| Y02 | 0.058 | ND | 100 |
| | 0.106 | ND | 100 |
| | 0.314 | ND | 100 |
| K08 | ND | ND | n/a |
| S07 | ND | ND | n/a |

ND=below the limit of detection, ~1 pg/μl. Samples refer to individual donors. Each row within a single donor shows results from replicate experiments performed on different days.

Table 3. Fluorescence Histogram Statistics for Contributor Cell Populations Shown in Figure 2

Fig 1a

| <i>Donor</i> | <i>Mean</i> | <i>Median</i> | <i># Events</i> ² |
|--------------|-------------|---------------|------------------------------|
| A42 | 540 | 427 | 3903 |
| B17 | 743 | 556 | 4625 |
| D02 | 305 | 212 | 5158 |
| J16 | 2606 | 2024 | 6475 |

Fig 1b

| <i>Donor</i> | <i>Mean</i> | <i>Median</i> | <i># Events</i> |
|--------------|-------------|---------------|-----------------|
| I66 | 341 | 253 | 1573 |
| J16 | 996 | 842 | 3375 |
| R12 | 497 | 252 | 599 |
| S07 | 236 | 177 | 2497 |

Fig 1c

| <i>Donor</i> | <i>Mean</i> | <i>Median</i> | <i># Events</i> |
|--------------|-------------|---------------|-----------------|
| D02 | 208 | 160 | 3653 |
| I66 | 372 | 276 | 1983 |
| J16 | 635 | 491 | 3767 |
| R12 | 469 | 298 | 1090 |
| S07 | 279 | 226 | 3751 |

Fig 1d

| <i>Donor</i> | <i>Mean</i> | <i>Median</i> | <i># Events</i> |
|--------------|-------------|---------------|-----------------|
| A42 | 959 | 554 | 4320 |
| B17 | 409 | 307 | 7727 |
| D02 | 1114 | 907 | 3524 |
| I66 | 314 | 244 | 5014 |
| J16 | 1245 | 982 | 4702 |
| R12 | 457 | 260 | 861 |
| S07 | 376 | 277 | 4676 |

Fig 1e

| <i>Donor</i> | <i>Mean</i> | <i>Median</i> | <i># Events</i> |
|--------------|-------------|---------------|-----------------|
| B17 | 349 | 280 | 3665 |
| D02 | 362 | 287 | 3041 |
| J16 | 589 | 515 | 1156 |
| R12 | 302 | 208 | 493 |
| S07 | 259 | 190 | 2028 |
| D11 | 276 | 220 | 4230 |

Fig 1f

| <i>Donor</i> | <i>Mean</i> | <i>Median</i> | <i># Events</i> |
|--------------|-------------|---------------|-----------------|
| J16a | 2606 | 2024 | 6475 |
| J16b | 635 | 491 | 3767 |
| J16c | 589 | 515 | 1156 |
| J16d | 996 | 842 | 3375 |
| J16e | 1245 | 982 | 4702 |

¹ Data is organized according to the histogram overlays shown in Figure 2. Mean (arithmetic) and median values are in relative fluorescent units (RFUs).

² Flow cytometry cell ‘events’ correspond to populations within FSC and SSC gates that select for intact epidermal cells.

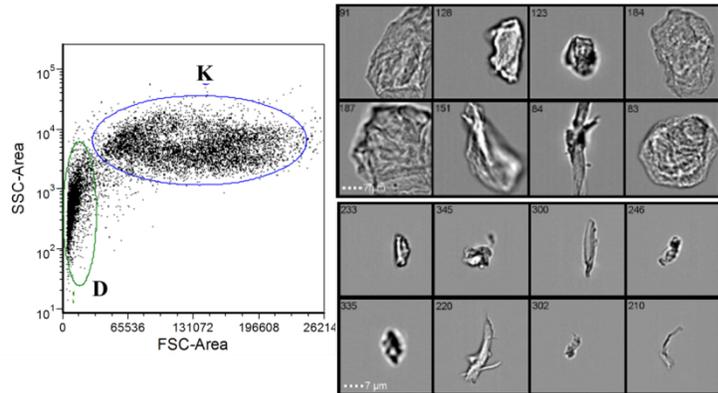


Figure 1. Optical characterization of a touch cell solution. Forward scatter and side scatter plot of all cell events showing ‘K’ and ‘D’ subpopulations (left). Images of individual cell events using AMNIS instrumentation (right). The top two rows are sampled from the K subpopulation and the bottom two rows are from the ‘D’ subpopulation.

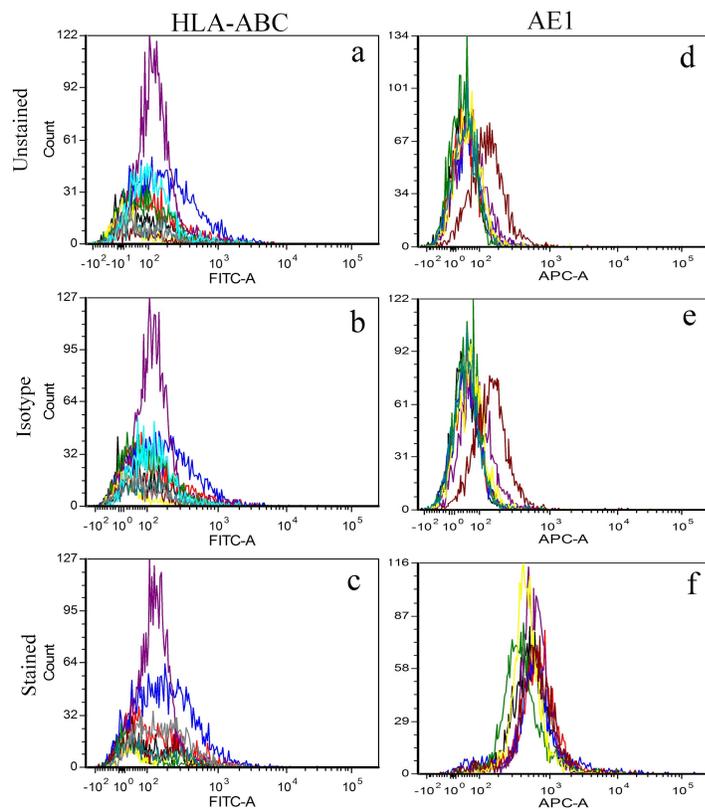


Figure 2. Hybridization of touch samples with HLA and CK antibody probes. Few differences were observed between samples hybridized with pan-HLA probe and unstained samples/isotype controls, indicating that the touch samples failed to uptake the probe (panels a-c). In contrast, all touch samples exhibited uptake of AE1 cytokeratin antibody probe, with slight differences observed in binding efficiency across contributor cell populations (panels d-f).

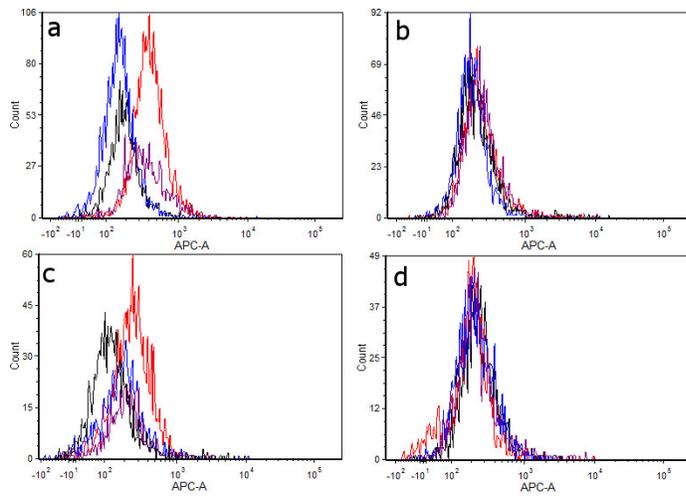


Figure 3. Replicate hybridization experiments using AE1 antibody probe. Touch samples were collected from the same four donors on four different days. On two of the days, differences were observed in the fluorescence profiles exhibited by from cell populations from was observed between donors (a, c). The same differences were not observed for two additional replicate experiments (b, d). Each of the four histogram colors is assigned to a separate contributor cell sample. The same four contributors were examined in each experiment.

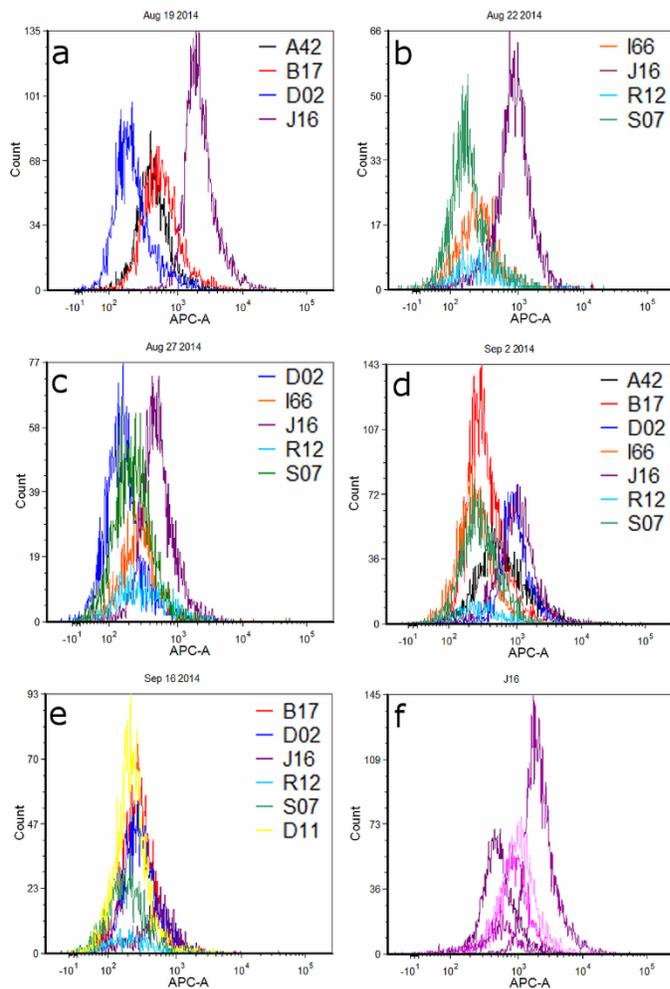


Figure 4. Overlaid red fluorescence channel histograms for epidermal cell populations from touch samples. Panels a-e show different combinations of donors cell populations each sampled and analyzed on the same day. Figure 1f is a histogram overlay of cell populations from contributor J16 across five different experiments.

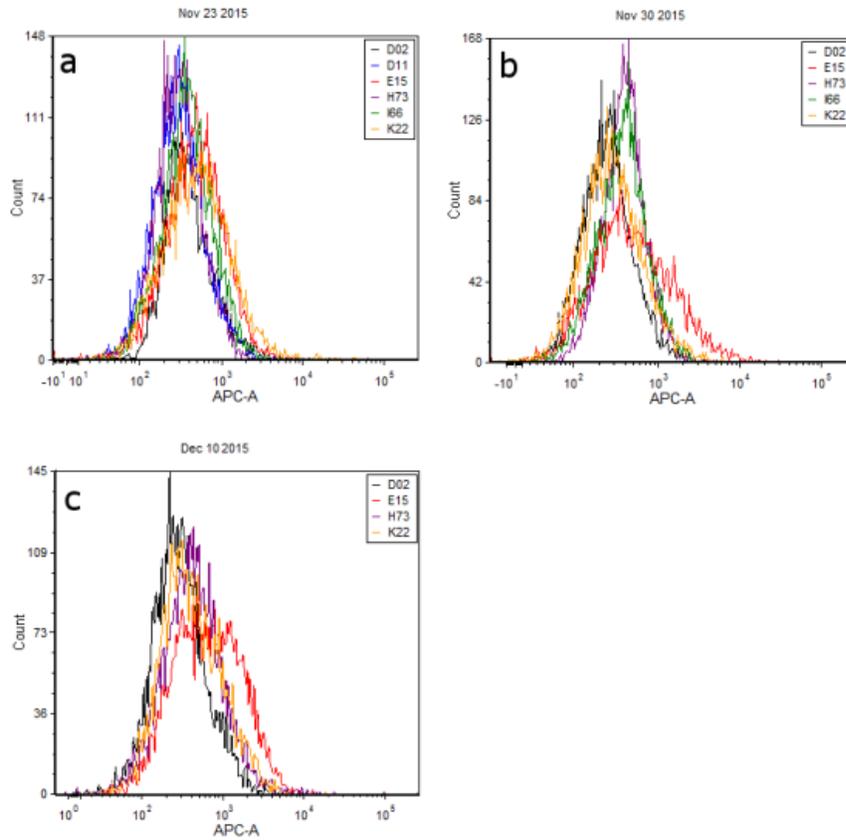


Figure 5. Overlaid red fluorescence (650-670nm) histograms for cell populations from touch samples. Each panel (a-c) shows a different combination of donor cell populations sampled and analyzed on the same day.

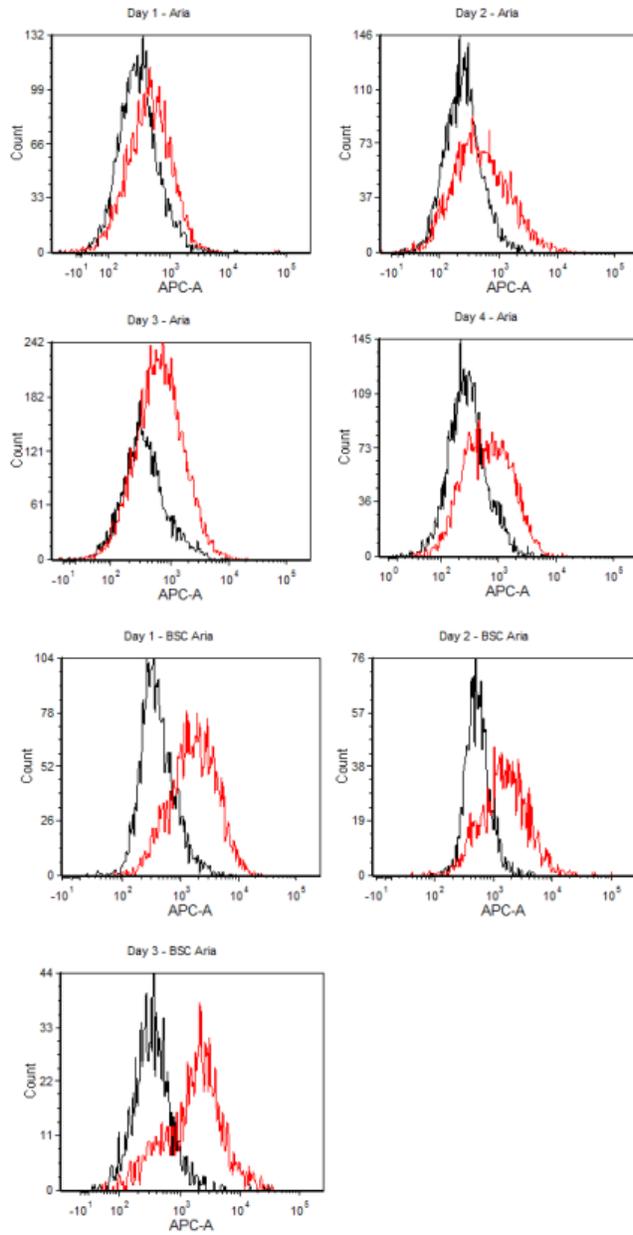


Figure 6. Overlaid red fluorescence histograms for two contributors, D02 (black) and E15 (red), across seven independent sampling days.

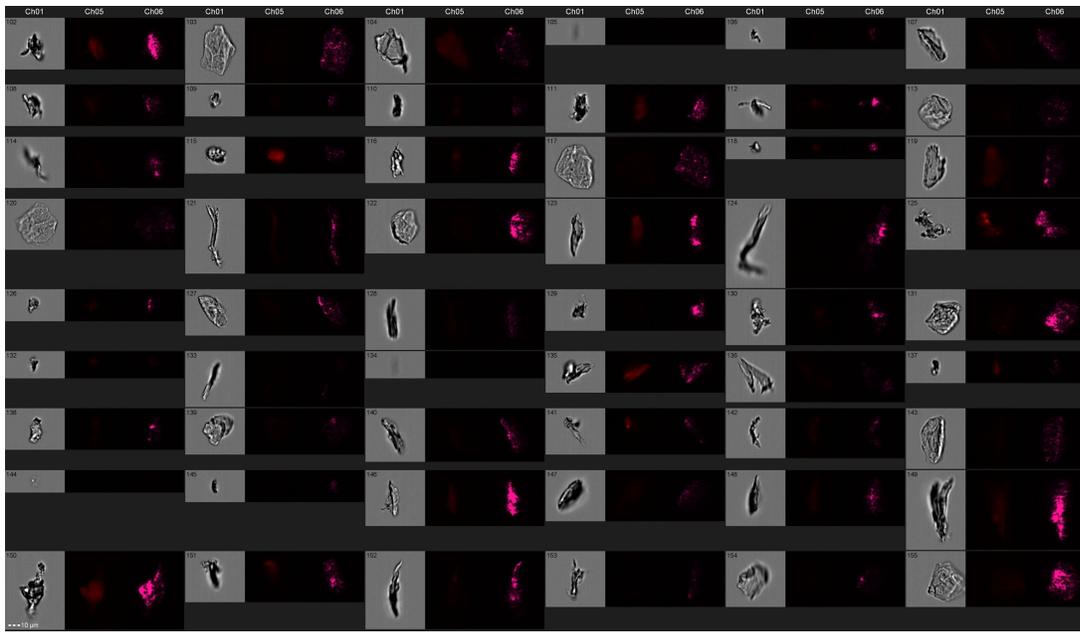
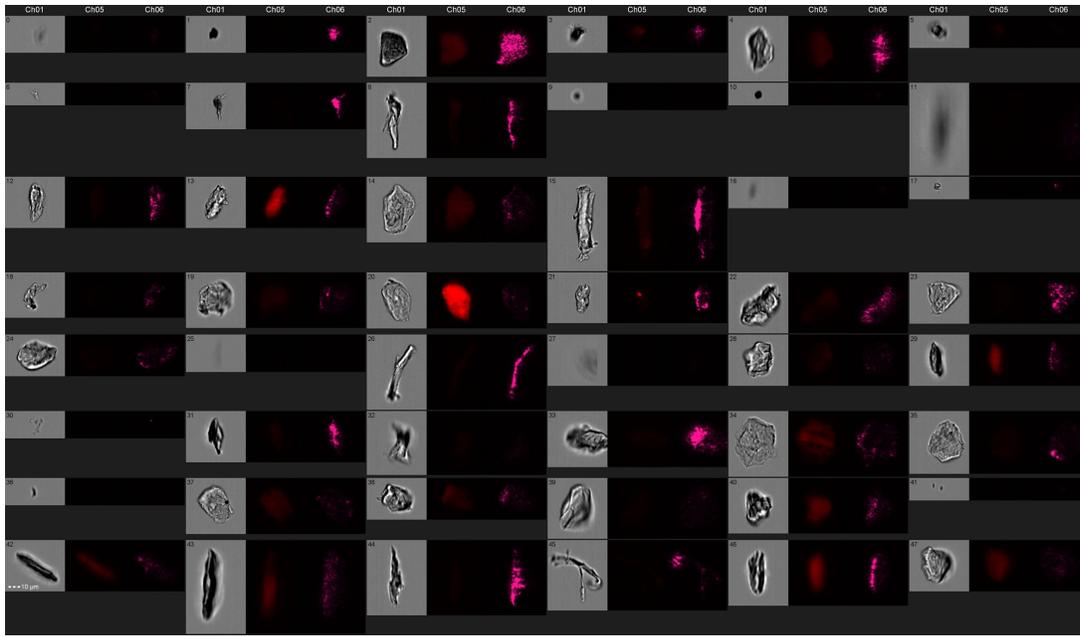


Figure 7. AMNIS imaging of individual flow cytometry events from the large (“K”) fraction of touch samples from two different contributors, E15 (top) and D02 (bottom). Each event was visualized in three different microscopic settings: Brightfield (left image in gray), APC channel fluorescence (middle image shown in red), and side scatter (right image shown in purple).

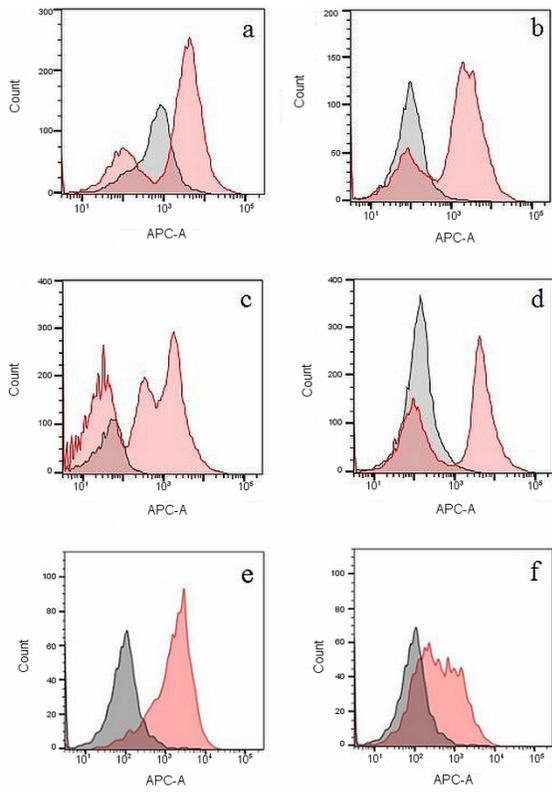


Figure 8. Overlaid APC-channel histograms for samples generated from donors handling plant material (kale or collard green leaves) prior to cell deposition. Each panel show cell populations from the right and left hand of four different contributors that handled plant material with only one hand (red histogram) leaving the other hand as a control (black histogram).

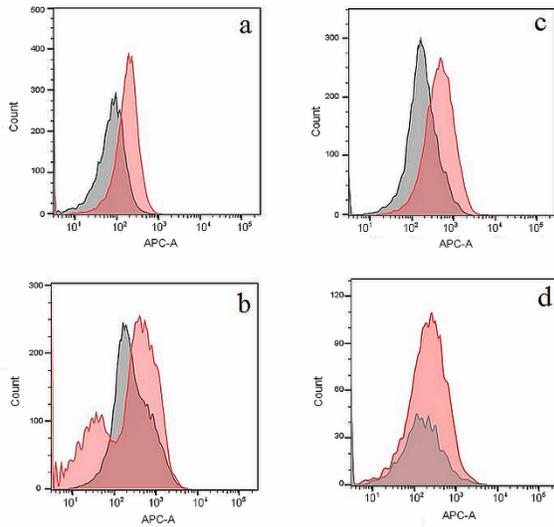


Figure 9. Overlaid APC-channel histograms for cell populations generated from the donors that wore a purple nitrile glove on one hand. The red histogram shows cells derived from the hand that had worn a purple nitrile glove with the gray histogram showing cell populations from the opposite hand that wore nothing (negative control). Panels (a)-(c) represent three different contributors. Panels (c) and (d) are experimental replicates of the same contributor sampled on different days.

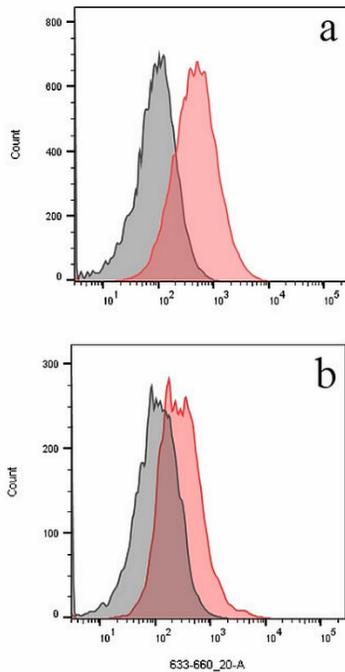


Figure 10. Overlaid APC-channel histograms for samples generated from donors handling substrates bearing marker ink prior to cell deposition. Panels a and b show cell populations from the right and left hand of the same contributor sampled on two different days that handled a substrate previously written on with marker. Only one hand (red histogram) handled marked substrate leaving the other hand as a control (gray histogram).

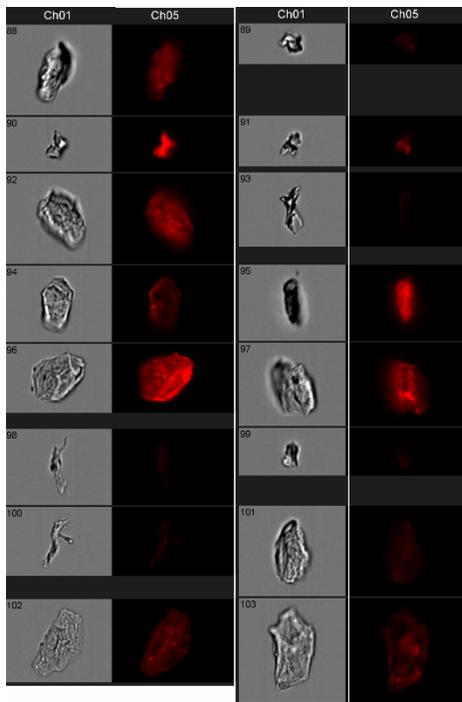


Figure 11. AMNIS imaging of individual flow cytometry events from the fluorescent cell population shown in Figure 3a (red histogram). Each event was visualized in two different microscopic settings: Brightfield (left image in gray) and APC channel fluorescence (right image in black background).

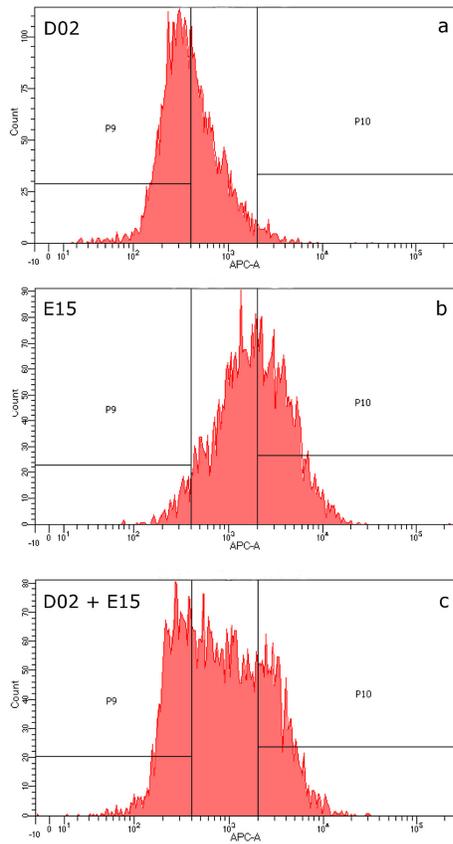


Figure 12. Sorting gates used for FACS based on APC channel intrinsic fluorescence. Histogram profiles for single source samples (panels a, b) were used to define two sorting gates, P9 and P10. These gates were positioned such that cell populations from D02 and E15 would be enriched relative to each other in the two cell fractions. Panel c shows the sorting gates plotted against the histogram profile of the two-person cell mixture prior to sorting.

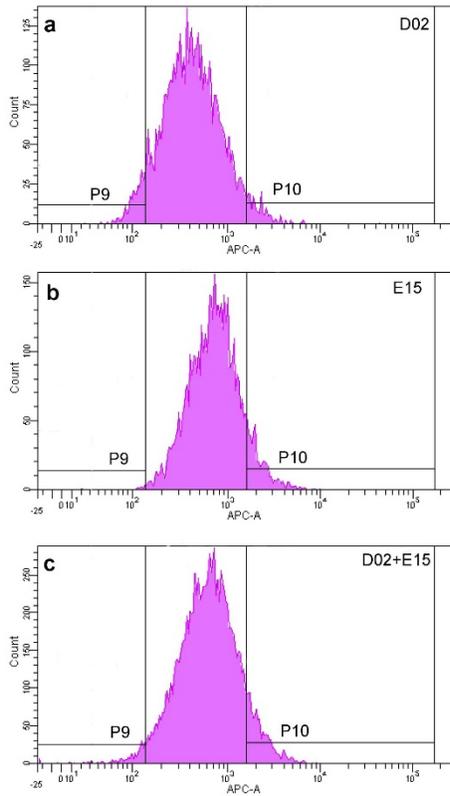


Figure 13. APC-channel histograms of touch samples from donor D02 (a), donor E15 (b), and mixture of touch samples from D02 and E15 (c). This mixture was created on a day when the fluorescence histograms of touch samples from D02 and E15 displayed a high degree of overlap. Histograms (a) and (b) were used to define two sorting gates, P9 and P10, designed to enrich cells from D02 and E15 (respectively). Panel c shows the more unimodal fluorescence histogram profile of the mixed sample (compared to histogram distribution observed in Figure 8c), overlaid with the pre-defined gates. Because most of the cells from D02 and E15 possess overlapping fluorescence characteristics, these gates capture a small fraction of the total sample (much smaller than the gates displayed in Fig. 8).

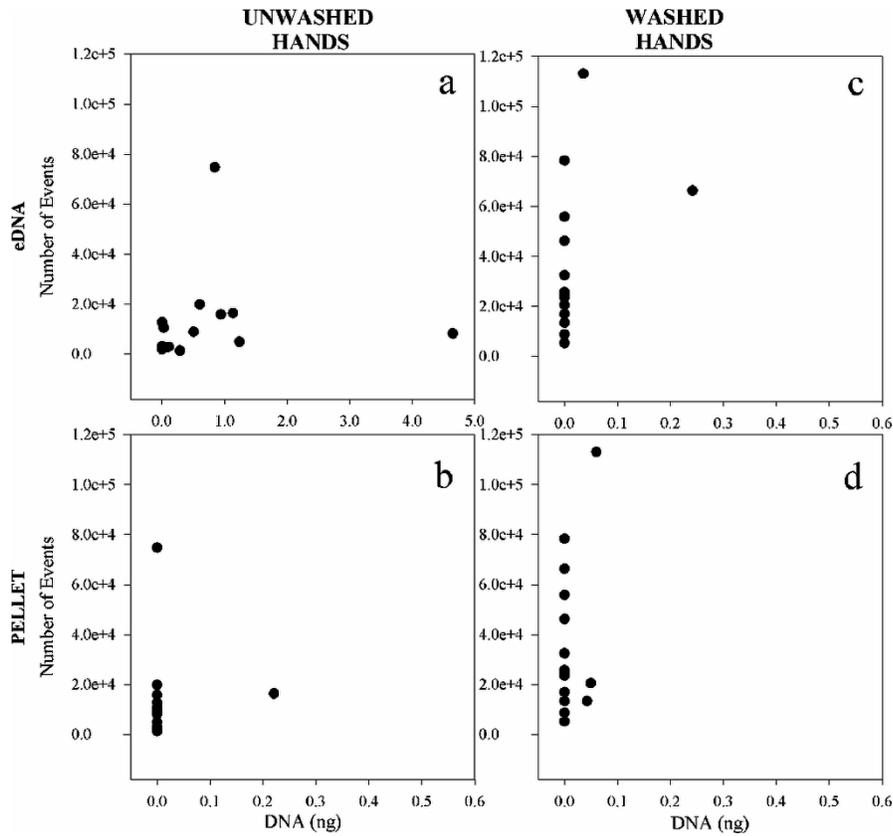


Figure 14. Cell counts and DNA yields from touch samples from washed and unwashed hands. For each graph, the Y axis represents the number of “K events” (cells) detected in solution from collection swabs (unwashed hands in a and b; washed hands in c and d), while the X axis represents the number of nanograms of DNA recovered (from supernatant (a) and cell pellet (b) of unwashed hands, and from supernatant (c) and cell pellet (d) of washed hands).

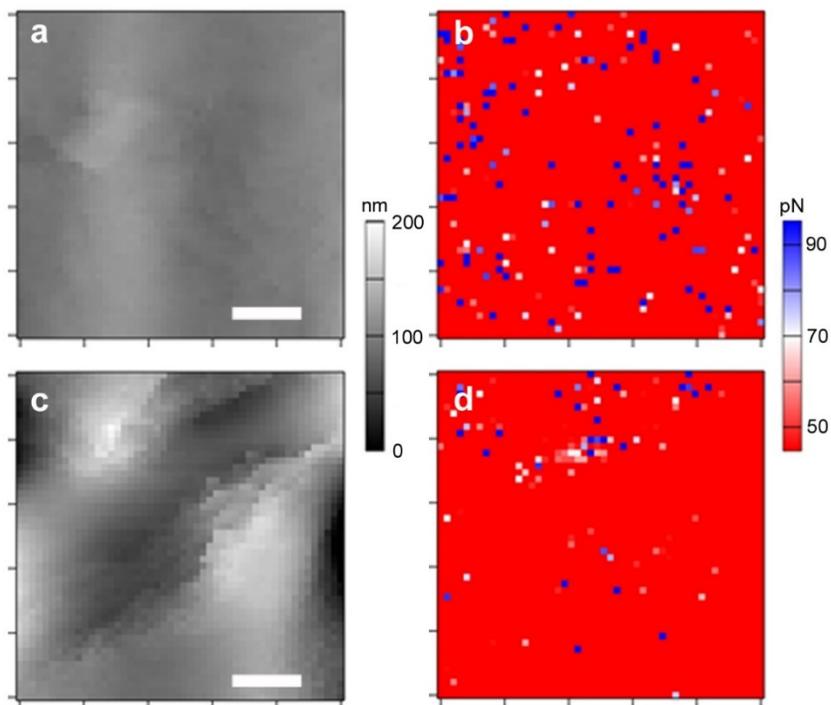


Figure 15. DNA force mapping of small areas of buccal cells (a, height map. b, force map) and palm cells (c, height map. d, force map.). The white to dark points on the force map indicate areas of increasing interaction of the lactoferrin with the surface (gray = minimal or no interaction), implying DNA presence. Scale bars: 1 μm on all images

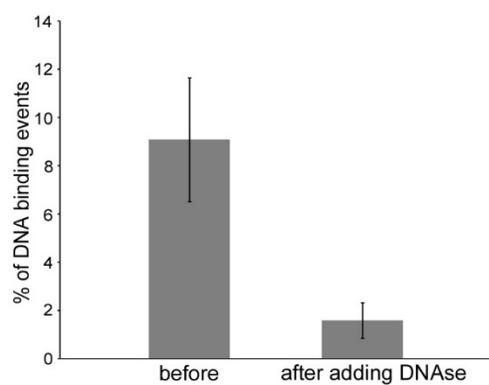
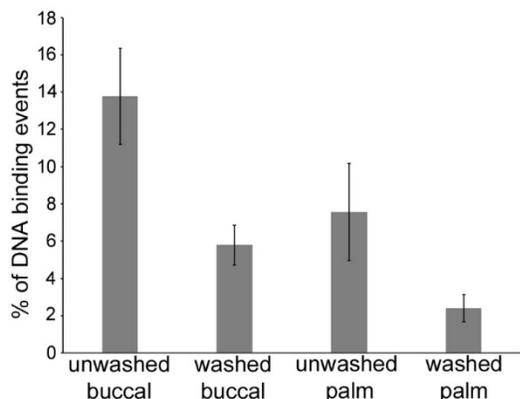


Figure 16: Aggregate data on DNA mapping on buccal and palm cells using lactoferrin. Cells from three donors - Three individual cells were analyzed for each donor (b) Control experiment: real time detection of DNA on unwashed palm cells before and after DNase incubation.

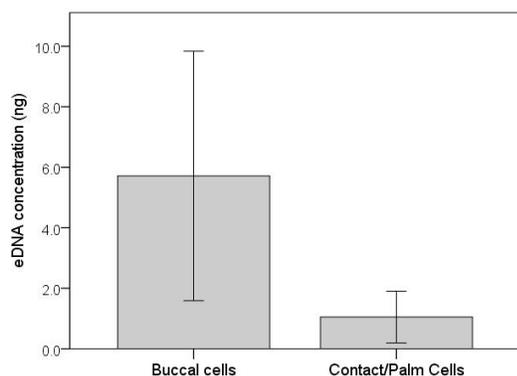


Figure 17. Preliminary survey of extracellular DNA associated buccal cells (n=3 donors) and contact epithelial cells (n=5 donors). Extracellular DNA was isolated and quantified using the same methods described in (Stanciu et al, 2015). Error bars represent one standard deviation.

IV. Conclusions

A. Discussion of Findings

The objective of this study was to characterize the optical and biochemical properties of touch epidermal cell samples and investigate different cellular properties that may be used to differentiate contributor cell populations in a touch mixture. Flow cytometry data showed that biological material recovered from standard sampling swabs and eluted in solution was composed of intact cells consistent in size with corneocytes (20-40 μ m) and smaller, irregular events. Single cell imaging of the latter fraction suggests that it is composed of cellular debris, deformed/damaged cells, and fiber fragments that may originate from the collection swab or are associated with the sampled substrate. Over the course of this study we observed both inter- and intra-contributor variation in the number of corneocytes detected and their percentage relative to the total number of events in a touch sample, consistent with previous reports. Nonetheless, cell yield was not an issue as touch swabs routinely provided more than 10,000 cells for analysis.

As with our previous studies of controlled touch samples, evidence of other epithelial cell types was not observed or detected (e.g., buccal cells which generally appear larger than corneocytes), although we note that damaged or fragmented cells from other tissues may be difficult to detect with these techniques. Since cell source information can be probative in some cases (e.g. to support or refute allegations of oral contact), future research should focus on methods of differentiating and identifying different epithelial cell types. As discussed further below, the same classes of proteins that we surveyed in these studies could potentially be used for this purpose (35), and possibly integrated with other cell targets and/or properties into the kind of flow/FACS methodology that we investigate here, thus permitting simultaneous discrimination between cell types and contributors to a mixture.

We hybridized epidermal cells against two different classes of antibody probe in order to assess whether the target proteins' variable expression had the potential to differentiate donor cell populations in a touch mixture. Hybridization experiments targeting HLA antigens on the cell surface showed little to no binding to either allele-specific or class-level antibody probes, suggesting that HLA antigens were either not present or were unreactive (Figure 2). The absence of HLA probe interactions in this study is further evidence that the overwhelming majority of cells in these touch samples are fully differentiated keratinocytes, which have been shown to display limited reactivity to HLA Class I probes in contrast to cells derived from deeper layers of the epidermis (30,31) or non-epidermal epithelial cell sources (36).

Of course, there is no such thing as a representative touch sample, and likely some touch samples encountered in casework will include non-corneocyte components such as buccal cells (37), which may prove reactive to cell surface probes. However, we did not detect any such cells in this study which may be characteristic of many of the touch samples recovered in case work. Regardless, before abandoning cell surface antigen targets such as these in touch samples, it may be worth exploring techniques such as preliminary trypsinization to increase immunoreactivity of corneocytes (30,31).

For purposes of the current studies, though, we moved on from HLA probes to test an antibody probe system that targets cytokeratins, which are an important structural component of both differentiating and fully differentiated epidermal cells (28). Specifically, we utilized AE1 probe which binds to cytokeratin proteins 10, 14, 15, 16, and 19. We found that touch samples consistently hybridized to the AE1 probe, albeit donors displayed slight variation in binding affinity (Figs. 2, 3). Across sampling days, the degree of variation occasionally increased (Figs. 3a and 3c), however, we observed that the difference was sometimes minimal (Fig. 2, Fig 3b and

3d). These results suggest that cytokeratin expression – at least on the pan-level that is capable of being explored with a probe such as AE1 – may not present a consistently useful means of discriminating between individuals. However, individual CK probes may prove more discriminating than pan probes, e.g., certain cytokeratins are upregulated, and others downregulated, with age (38). By targeting cytokeratins on a pan level, these differences may be cancelled out. Expression of individual CK proteins has also been used to distinguish between epithelial cell sources (mucosal epithelial cells (buccal or vaginal) from epidermal cells) (35), and could possibly be used in conjunction with flow cytometry to detect the presence of, and potentially isolate, non-epidermal cell types in touch samples (or, background levels of skin cells in a non-touch sample).

Our observation in the course of antibody hybridization studies that intrinsic fluorescence – particularly at red wavelengths – varied between donors led us to pursue this feature for its potential in discriminating between cell populations. Results from initial experiments involving eight donors revealed clear differences between certain pairs of donor fluorescence profiles (Figure 4; Table 3), such that fluorescence-based sorting gates could be conceived that would isolate cells from one or more contributors, to the exclusion or minimal contribution of cells from others. In subsequent experiments, less distinction was observed between donors (Fig. 5), and it is unclear whether or how much of this may be attributable to differences in instrumentation (Figure 4 generated using BD Canto platform while data shown in Figure 5 was generated from two different FACS Aria instruments; further, the voltage settings for the two sets of experiments differed), the specific donors tested (e.g. J16, the donor who exhibited the highest levels of red fluorescence in the earlier studies, was not available during for this study), or possibly a combination of these factors. The influence and potential impact of day-to-day

sample variation cannot be discounted, particularly where previous studies also found fluctuation in fluorescence measurements for a donor whose cells were sampled on multiple days and analyzed on a single instrument (Figure 4f).

Regardless, in the current study, one donor in particular consistently exhibited higher red fluorescence than other donors (E15 histograms in Figs. 5 and 6). On some days, this donor's touch cell populations exhibited autofluorescence several magnitudes greater than other days: across seven sampling days, median autofluorescence for E15's touch samples ranged from ~500 to 3000 RFUs. On the days when E15's touch samples emitted the highest red fluorescence, the degree of differentiation from other donors' cell populations, in particular D02, was the greatest (Fig. 6).

Understanding the factors, both intrinsic and extrinsic to the cell, which may cause shifts in autofluorescence will be an important area of future research. There are a number of endogenous molecules within the stratum corneum that can contribute to autofluorescence (39), including molecules such as porphyrins which have emission maxima similar to what was observed in this study (40,41). Although microscopic surveys are consistent with some portion of the red autofluorescence signal being associated with apparent corneocytes (Figure 7), we also noted that other, likely non-cellular, fluorescent particles could be found in these samples and may contribute to the overall optical profiles. These included particles consistent with hair fragments or fibers that were recovered from touch samples of multiple donors. The fact that both cellular and non-cellular material was observed to fluoresce also raises the possibility that exogenous fluorescent compounds (e.g., plastics (42), chlorophyll (43), or inorganic molecules) may associate with (and thus be transferred with) cellular (and non-cellular) material on the palms and contribute to a touch sample's autofluorescence.

Subsequent experiments testing this hypothesis demonstrated that an individual's immediate prior contact with exogenous substances can significantly affect the fluorescence profile of the ensuing touch cell populations. Fluorescent compounds from handled materials may be transferred to and essentially "tag" cells of the palm, and in turn may be transferred, in association with those cells, to touched objects. The known fluorescent properties of aromatic chlorophylls make these compounds a compelling candidate for the source of observed autofluorescence in experiments involving plant material. It should be noted that contributor cell populations in Figure 4 also exhibited fluorescence at shorter wavelengths (*e.g.*, 488nm), consistent with chlorophyll compounds which exhibit autofluorescence across a wide range of wavelengths (43).

The compound responsible for the less pronounced increase in red autofluorescence intensity observed in experiments involving purple nitrile gloves is more ambiguous. Notably, we investigated this phenomenon further by having donors wear other gloves found in our laboratory, including blue nitrile and latex; associated touch samples did not exhibit increased red fluorescence. This may indicate that there is some substance unique to the purple nitrile gloves or that particular commercial brand that is the source of observed fluorescence. Laboratory gloves may be treated with a variety of additives, synthetic preservatives, or antimicrobials to facilitate removal, maintain shelf life and/or ensure sterility (44) which may potentially impart fluorescence. Similarly, colored marker ink may contain a variety of components that contribute to fluorescence emissions within the visible spectrum including dyes, pigments, and fluorescers (45) which has been investigated within forensic contexts (46).

The day-to-day profile variability shown in Figure 4 (where no particular materials were deliberately handled) suggests that there may be multiple factors or compounds contributing to

the red autofluorescence signature. The frequency, duration, and type of contact a contributor has with various exogenous substances is likely to dictate the intensity and persistence of red autofluorescence (as well as autofluorescence at other wavelengths) in cells left by touch. We also cannot discount the possibility that differences in epidermal cell biology or palmar characteristics (e.g. sweat levels) between individuals may contribute to the persistence of exogenous materials on keratinocyte populations.

While the above experiments suggest that a donor's contact with specific substances may influence the fluorescent properties of subsequently sloughed keratinocyte cell populations, this does not preclude the possibility that intracellular components may also contribute to this effect, as discussed previously (47). Understanding the factors, both intrinsic and extrinsic to the cell, which may cause shifts in autofluorescence in touch cell populations will be an important area of future research.

Ultimately, our observations regarding variations in red fluorescence in touch deposits suggest that there will be some touch cell mixtures that are more susceptible to being separated into individual components (or at least broken down into less complex cell mixtures) based upon this characteristic than others. Because flow cytometry is non-destructive, evidence samples could potentially be screened for favorable fluorescence distributions. A mixed cell sample that exhibits two or more peaks (e.g. Fig. 12) on a fluorescence histogram may be a more promising candidate for cell separation than one that exhibits a unimodal fluorescence distribution (e.g. Fig. 13), as the latter suggests a high degree of overlap between contributor cell populations. Our preliminary results appear to bear out this proposition, but further research is required to develop a standardized set of screening criteria.

However, even a touch sample composed of readily-distinguished cell populations will not necessarily separate cleanly, or produce worthwhile STR data. Our group and others have reported on the characteristically low levels of intracellular genomic DNA recovered from cells deposited on touch surfaces (33,48), which is expected given that keratinocyte differentiation involves programmed breakdown of nuclear DNA prior to cell shedding from the stratum corneum (26,49). This could pose a challenge for the application of cell-based separation techniques on touch samples. With that in mind, we utilized autofluorescent signatures to sort a controlled touch mixture of donors D02 and E15 via FACS and attempted DNA analysis of the resultant fractions using a standard forensic workflow.

Our preliminary efforts resulted in a partial STR profile for each sorted touch fraction that is (with the exception of a single extraneous allele) consistent with the respective known contributor, indicating that separation of cell populations from the two known contributors on the basis of red autofluorescence was successful. However, the single stray allele in each sort suggests that a very low level of DNA from a third party may have ended up in these fractions. Given the low levels of target template, it is possible that these are examples of allelic drop in during amplification; negative controls were clean but this does not exclude the possibility of this phenomenon. Interestingly, six extraneous alleles (i.e. not from D02 or E15) were detected in the reference (unsorted) mixture (Table 1). None of these alleles showed up in profiles developed from Sort A or Sort B. These could be instances of drop in (11.3 at D2S441, 20 at D18S51 and 8 at D8S1179) and pronounced stutter (17 at D2S1338, 14 at D16S539, and 23 at FGA) resulting from low levels of DNA template in the touch mixture. It is also possible that these alleles are derived from extracellular DNA (which would not be expected to show up in

sorted fractions) that was transferred to the palms of D02 or E15 before they deposited their touch samples, particularly in light of studies demonstrating the prevalence of extracellular DNA in touch samples (33,48,50).

The high degree of allelic dropout observed in the sorted fractions is not unexpected given the nature of the biological material being analyzed – shed epidermal cells. However, there are several areas in our methodology where adjustments could be made to improve DNA yield and/or maximize the use of the DNA that is present, and thus produce more complete DNA profiles from sorted fractions. For example, we utilized a standard forensic DNA analysis protocol on sorted samples, which could be modified in various ways to increase efficiency (e.g. by reducing extract volume and/or concentrating post quantitation). Moreover, these controlled touch mixtures were split into aliquots to be used for differing purposes during these exploratory studies (e.g. microscopic imaging, FACS, DNA analysis without sorting). As such, only a fraction of the cells collected from touched surfaces were submitted to FACS; if more (or all) of the touch samples were utilized for this purpose, each fraction would likely contain more cells for downstream STR profiling.

Further, by designing the sorting gates in this study with an eye toward producing single source profiles, we sacrificed maximal cell recovery for purity of the sort. As can be seen from Figure 6, gate P9 was designed to capture D02's cells while excluding most of E15's cells, and gate P10 was designed to capture E15's cells while excluding most of D02's. However, approximately half of each of D02 and E15's cells went unsorted in the middle area between the two gates. With touch samples, and the associated difficulties related to intracellular DNA yield from corneocytes, it may make sense to shift the gating calculus we used for other types of biological material (24). Instead of designing gates to produce single source profiles, one might

strike a balance between cell recovery and production of simple mixtures with easily discernable major components.

For example, if the gates in Figure 12 were set so that all cells in the D02-E15 mixture fluorescing less than 1000 RFU were sorted into Sort A, and those fluorescing at or greater than 1000 RFU were sorted into Sort B, this should result in recovery of all cells from the mixture between the two fractions. Note that while most of D02's cells exhibit fluorescence below 1000 RFUs, a few cells fluoresce at a higher intensity (Fig 12a); conversely, while most of E15's cells exhibit fluorescence above 1000 RFUs, a few cells fluoresce at a lower intensity (Fig 12b). Thus, while each fraction sorted in this manner will contain some cells from the untargeted contributor, resulting in a mixture, the major contributor should be distinguishable and consistent with the vast majority of cells in the simplified mixture created by the sort (D02 in Sort A; E15 in Sort B).

One of the biggest drivers of cell loss in our methodology may be the retention of cellular material in the collection swabs following manually stirring and vortexing in water to elute the cells into solution. The challenge of maximizing DNA yield from collection swabs has been explored by a number of researchers in the forensic sciences, though many of the protocols are not applicable where, as here, cells need to remain intact during elution (51). Future work should continue to test different elution protocols to maximize cell recovery; optimized buffers (52) and the incorporation of enzymes such as cellulase to break down cotton and encourage the release of cells (53) may hold promise. To the extent that some number of cells will undoubtedly remain trapped despite methodological adjustments, subsequent studies should investigate whether and how information derived from this biological material may be exploited. At very least, this unsorted mixture data may be used to give context to STR profiles developed from sorted cell

fractions; in some cases, the combination of sorted and unsorted DNA data may increase the overall probative value of a sample.

Finally, because a significant portion of the genetic material in many touch samples may be unavoidably extracellular, characterizing the chemical and physical relationship between cell-free DNA and the surface of intact epidermal cells may be an important area of future research. If extracellular DNA associates with epidermal cells, as it has been observed to do in other cell types (e.g., (54)), flow cytometry protocols could potentially be optimized to maintain surface-bound DNA through the cell sorting process. If it emerges that extracellular DNA is not bound to epidermal cells at the time of transfer, this DNA source can be separately collected for typing (33).

Quantitative Relationship between Epidermal Cells and Extracellular DNA in Touch Samples

Our results contribute to the forensic community's growing body of knowledge on touch samples. We found that the vast majority (~84-100%) of nuclear DNA recovered from touch samples collected under the conditions described above is extracellular. Amplifiable DNA from the pelleted cellular fraction was detected in only eight of the 51 touch samples analyzed (Fig. 14, Table 3).

Although this finding is generally consistent with other recent studies suggesting the significance of extracellular DNA in touch evidence (48,50), the prevalence and proportion of extracellular DNA relative to the total DNA yield shown in Table 1 was higher than observed in other studies (50). It is possible that the multiple wash steps performed on the pelleted cell material for this study removed more eDNA than efforts utilizing a single wash. In a separate analysis of seven replicate samples, we found that additional eDNA was often recovered with

additional wash steps, and concurrently, that a systematic cell loss at each wash step was not observed—a Student’s t-test on cell counts before and after three wash steps yielded an average p-value of 0.28 with only two of the individual replicates yielding p-values less than 0.01. This suggests that while some cells may have been unintentionally removed from some cell pellets by our methodology, this phenomenon is unlikely to explain the consistent increased DNA recovery in the supernatant with additional washes across samples.

The nature of the samples likely played a role as well, as there may have been more opportunities to pick up nucleated cells for some casework samples described in other research (50) than our controlled conditions. The fact that the “typical” or “standard” touch sample evades definition poses a challenge when designing studies to better understand these kinds of samples. It has been suggested that saliva, which contains buccal cells, may be an important (*i.e.*, DNA rich) component of some touch samples (37). We observed no evidence of such cells – which generally appear larger than corneocytes (>60 μm for buccal cells versus 20-40 μm for corneocytes) – in microscopic surveys of individual cells within two touch samples. However, this does not preclude the possibility that non epidermal cells were present, since only a portion of the sample was surveyed, and because deformed or fragmented cells from different tissues may be indistinguishable from corneocytes. Future work could explicitly test for the presence of buccal cells in touch samples through, *e.g.*, antibody hybridizations targeting tissue specific surface antigens coupled with flow cytometry.

The mechanism of touching could also affect the proportion of eDNA to iDNA in touch samples; our preliminary data from touch samples deposited by rubbing suggest that this action may result in considerably higher cell pellet yields than samples deposited by holding, perhaps by exposing deeper (*i.e.*, undifferentiated) layers of cells. However, in these preliminary

experiments we also observed that the amount of eDNA left by rubbing the substrate was similar to levels of eDNA left by holding. This suggests that the transfer of eDNA may not be as affected by the manner in which a substrate was handled as iDNA transfer.

In any case, our results lend further support to the concept that extracellular DNA is particularly crucial to the analysis of touch samples. Measures should be explored to exploit this source of information to the greatest extent possible. For sample collection and processing purposes, this may dictate that touch samples be treated differently than other types of forensic biological sample. To avoid the significant loss of DNA that may be associated with extraction, it may make sense to process the eDNA-containing supernatant separately via direct amplification; our results suggest that care should be taken to maximize the amount of eDNA washed into the supernatant.

Our finding that the number of cells in touch samples was uncorrelated to the amount of extracellular DNA or the total DNA yield suggests that not only is the recoverable DNA primarily extracellular but that it is not immediately derived from the large numbers of epidermal cells that are shed daily. DNA was not detected in the cell pellet of samples that contained more than 100,000 cells, while samples comprised of far fewer cells (~2000) yielded DNA. Our extraction methodology likely had some impact on overall DNA yield (55); we have found in other experiments that other extraction methodologies (*e.g.*, DNA IQ) resulted in low (<80pg) but quantifiable DNA yields in samples that yielded no DNA after processing with the extraction method utilized here. However, this does not change the fact that a considerable portion of DNA from the touch samples that we analyzed was extracellular, and that the number of cells shed was not a reliable indicator of DNA yield. These results are compatible with previous medical research showing that corneocytes from the outermost epidermal layer (*i.e.*,

stratum corneum) have little to no genomic DNA owing to the controlled degradation of intracellular components during differentiation (56).

Accordingly, epidermal cells – even when present in large quantities – may make a fairly insignificant contribution to either intra- or extracellular DNA recovery from touch samples. Consistent with recent studies that found no evidence of fragmented DNA in the epidermal layers (in contrast to sebaceous cell sources) (57), the majority of extracellular DNA in touch samples is likely derived from alternate sources such as oil and sweat secretions, or saliva (8, 11). Where intracellular (*i.e.*, cell pellet) DNA levels from touch samples are considerably higher than those observed in this study, a nucleated cell source (*i.e.*, non-epidermal, or more basal epidermal) may be implicated, though certain skin conditions are known to result in the aberrant retention of nuclear DNA in corneocytes (56).

Although hand washing resulted in the transfer and subsequent recovery of little to no eDNA, we found that cells were nonetheless transferred. In fact, we observed greater levels of cellular transfer among washed hand samples than unwashed hand samples. It is possible that the act of hand washing loosens or sloughs off corneocytes, and that these cells (perhaps because of their flattened morphology) are more likely to persist through the washing process than eDNA. Regardless of the explanation, an estimated thousands to hundreds of thousands of cells survived the hand washing process to be transferred from the palmar surface by simple touching.

Consistent with Locard's principle, while these shed corneocytes may not contain sufficient levels of nuclear DNA to generate a probative STR profile, there is the possibility that other, non-genetic signatures could be analyzed, so that the most challenging touch samples (*i.e.* those that contain little to no DNA) may provide forensically relevant information. For example, the average size of individual corneocytes has been shown to vary with source factors such as

age, sex, and anatomical region (58,59), as does the composition of intracellular cytokeratin components (38). While further research is of course necessary to assess the degree of inter- and intra-individual variance in particular cellular features, determining such source attributes from unknown contributors could potentially provide leads or exclude suspects in specific types of investigations, e.g., sexual assault, molestation. Further, the absence of amplifiable nuclear DNA in corneocytes does not necessarily preclude the presence of sufficient levels of mitochondrial DNA to permit typing. Combining techniques to sort epidermal cells into donor populations (e.g., using factors described above) and typing the mtDNA of those populations is an avenue that warrants further exploration.

Analysis of extracellular DNA on ‘touch’ epithelial cells

Overall, our high resolution imaging analysis suggests that presence and relative quantity of surface-associated, extracellular DNA signatures can be analyzed on individual epithelial cells from different tissue sources. Control experiments using DNase treated cells indicate the majority of binding events represent probe interactions with DNA on the cell surface. Because interactions between lactoferrin probe and DNA are primarily electrostatic, a minor percentage of the binding events could be due to other negatively charged biopolymers on the cell surface (e.g., small percentage of probe binding events detected after DNase treatment). Nevertheless, the observed variation in DNA content across individual cells, cell types, and before and after washing suggest that this technique may be an effective strategy for examining contributor-specific and tissue-specific differences in eDNA content as well as the transfer and persistence of eDNA in various types of forensic samples. In considering the total *surface* DNA, despite the differences in keratinization, it appears that the presence of eDNA is dependent on the source of

the cells (individual-specific) rather than its location. It is important to note that touch DNA yields do tend to be extremely variable between replicates of the same person, so it is indeed difficult to draw a direct connection between relative amounts of DNA between the same individuals unless the same sample is used for both AFM and bulk genomic analysis. Our preliminary results indicate that cell surface DNA force mapping therefore has the *potential* to identify the spatial distribution across different donors, for instance, noting the fact that some individuals tend to have higher DNA content on their keratinized palm cells. We note that the hypothesis that some people are better DNA shedders than others, continues to be a subject of some debate. More multi-day replicates are needed to fully investigate these at both the bulk and single cell level to justify drawing any direct correlation between the two.

B. Implications for Policy and Practice

This investigative study serves as an important foundation for ongoing research into methods that facilitate the separation of touch samples into individual contributor cell populations for downstream DNA analysis. While additional research is needed before FACS can be imported as a front end technique in forensic DNA casework, our results indicate that there are features of fully differentiated keratinocytes (whether endogenous or exogenous is currently unclear) that can be harnessed to distinguish cell populations from some individuals. A benefit of a feature such as red autofluorescence is that it can be measured without the need for antibody probes or other special reagents, allowing for touch samples to be pre-screened for this trait. However, the recovery of even partial profiles from sorted cell solutions, as we have demonstrated here, may have the potential to enhance the overall probative value of DNA evidence, particularly when analyzed in conjunction with complex mixture data derived from the same sample (e.g. if it is combined with profiles generated from the extracellular fraction and/or

cells retained in swabs). Sorted profiles, even if too incomplete to stand alone, may be able to buttress probabilistic claims about the mixture. At very least, this data could provide important investigatory leads, e.g. by supplying clues as to allelic pairings in an otherwise indistinguishable mixture, and potentially narrow the pool of suspects.

Our investigations into the quantitative relationship between epidermal cells and DNA recovery from touch samples suggest that many traditional explanations of DNA analysis from touch samples used in expert testimony – which often seek to explain the quantity and quality of DNA detected (or lack thereof) in terms of an individual’s inherent or circumstantial susceptibility to shed epidermal cells – may need to be modified to reflect fundamental shifts in the forensic community’s understanding of touch evidence. Future research efforts should continue to examine the relationship between the transfer of eDNA, iDNA, and intact corneocytes onto touch surfaces by testing other types of depositional circumstances, e.g., different substrate material or touch samples from multiple donors.

C. Implications for Further Research

Results from this project establish a key foundation for continuing efforts focused on differentiation of contributor cell populations in a touch mixture and their separations. Understanding the factors, both intrinsic and extrinsic to the cell, which may cause shifts in autofluorescence will be an important area of future research. Additionally, work should continue to investigate intrinsic and/or environmental factors that can contribute to differences in cytokeratin expression, as well as whether shifts in CK expression as a function of age, cell source, and other factors can be detected and thus used (individually or in combination) to distinguish cell contributors in touch samples. Lastly, to help improve the recovery of full STR

profiles from sorted cell populations, future efforts should focus on testing a variety of DNA extraction and amplification conditions that are optimized for low template samples.

V. Bibliography

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VI. Dissemination of Research Findings

Peer-Reviewed Publications

- (1) Philpott, M.K., Stanciu, C.E., Kwon, Y., Bustamante, E.E., Greenspoon, S.A., **Ehrhardt, C.J.** 2017. Analysis of cellular autofluorescence in touch samples by flow cytometry: implications for the front end separation of trace mixture evidence. *Journal of Analytical and Bioanalytical Chemistry*. 409(17): 4167-4179. DOI: [10.1007/s00216-017-0364-0](https://doi.org/10.1007/s00216-017-0364-0).
- (2) Wang, C., **Ehrhardt, C.J.**, Yadavalli, V. Nanoscale characterization of forensically relevant epithelial cells and surface associated extracellular DNA. *Forensic Science International*. 277: 252-258. DOI: [10.1016/j.forsciint.2017.06.019](https://doi.org/10.1016/j.forsciint.2017.06.019)
- (3) Stanciu, C.E., Philpott, M.K., Bustamante, E.E., Kwon, Y.J., **Ehrhardt, C.J.** 2016. Analysis of red autofluorescence (650-670nm) in epidermal cell populations and its potential for distinguishing contributors to ‘touch’ biological samples. *F1000 Research*. 5:180. doi: [10.12688/f1000research.8036.1](https://doi.org/10.12688/f1000research.8036.1)
- (4) Stanciu, C.E., Philpott, M.K., Kwon, Y.J., Bustamante, E.E., Ehrhardt, C.J. 2015. Optical characterization of epidermal cells and their relationship to DNA recovery from touch samples. *F1000Research*, 4: 1360. doi:[10.12688/f1000research.7385.1](https://doi.org/10.12688/f1000research.7385.1).
- (5) Kwon, Y.J., Stanciu, C.E., Philpott, M.K., **Ehrhardt, C.J.** 2016. Flow cytometry dataset from cells collected from touch surfaces. *F1000 Research*. 5:390: doi: [10.12688/f1000research.8338.2](https://doi.org/10.12688/f1000research.8338.2).
- (6) Stanciu, C.E., Kwon, Y.J., **Ehrhardt, C.J.** 2016. Forward-scatter and side-scatter dataset for epithelial cells from touch samples analyzed by flow cytometry. *Data in Brief*. 6: 416-418. [doi:10.1016/j.dib.2015.12.027](https://doi.org/10.1016/j.dib.2015.12.027).

Conference Presentations

- (1) Ehrhardt, C.J. Optical differentiation of epithelial cell populations in contact cell mixtures using flow cytometry. Oral presentation. American Academy of Forensic Sciences, Las Vegas NV. Accessible at *F1000Research* 2016, 5:197. February, 2016. doi:[10.7490/f1000research.1111315.1](https://doi.org/10.7490/f1000research.1111315.1)
- (2) Stokes, N., Stanciu, C.E., Ehrhardt, C.J., Greenspoon, S.A. Cell separation of multiple contributor samples to facilitate DNA mixture analysis. Oral Presentation. American Academy of Forensic Science. Las Vegas, NV. February, 2016.
- (3) Stanciu, C.E., Kwon, Y.J., Bustamante, E.E., Philpott, M.K., Dawson Cruz, T., Ehrhardt, C.J. Differentiation of Individual Cell Populations in a Touch Mixture Using Antibody Probes and Flow Cytometry. Poster Presentation. International Symposium

Human Identification, Grapevine, TX. Accessible at *F1000 Research* 4:1371; doi [10.7490/f1000research.1111084.1](https://doi.org/10.7490/f1000research.1111084.1).

- (4) Stanciu, C., Kwon, Y., Ingram, S., Bustamante, E., Seashols, S., Dawson Cruz, T., Ehrhardt, C.J. Separation of Epithelial Cell Mixtures Using Fluorescently Labelled Antibodies and Flow Cytometry. Poster Presentation (Abstract #B13). AAFS, Orlando, FL. February, 2015. Accessible at *F1000Research* 2016, 5:107 doi:[10.7490/f1000research.1111278.1](https://doi.org/10.7490/f1000research.1111278.1)
- (5) Stanciu, C., Kwon, Y., Ehrhardt, C.J. Separation of Complex Biological Mixtures Using Human Leukocyte Antigen Probes and Fluorescence Activated Cell Sorting. International Symposium on Human Identification, Phoenix AZ, Poster Presentation, October, 2014.

Publicly Available Data Repositories

- (1) Ehrhardt, Christopher; Philpott, Kate (2016): Antibody Hybridization Dataset Using Human Leukocyte Antigen (HLA) Probes and Epithelial Cells (Buccal and Contact Epidermal). <https://dx.doi.org/10.6084/m9.figshare.3457265.v1>
- (2) Ehrhardt, Christopher; Philpott, Kate (2016): Antibody Hybridization Dataset with Cytokeratin Probes and Contact Epithelial Cells. <https://dx.doi.org/10.6084/m9.figshare.3444908.v1>
- (3) Ehrhardt, Christopher; Philpott, Kate (2016): Autofluorescence Dataset for Contact Epithelial Cells Analyzed by Flow Cytometry. <https://dx.doi.org/10.6084/m9.figshare.3463325.v1>