Final Summary Overview

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Project Title:
Front-end differentiation of contributor cell populations and estimation of DNA content using novel cellular signatures

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Purpose

The objective of this project is to develop a new method for screening trace biological samples for the number of contributors and DNA content based on the presence and relative abundance of key protein and hormone targets within cell populations. There is a critical need for presumptive techniques that could provide valuable information and enable more effective triaging of casework samples, particularly touch samples. Currently, 'blind' sampling of potential epithelial trace/touch samples as well as the often daunting mixture interpretation present many challenges for caseworking laboratories. To address this, we developed a novel workflow for analyzing biological evidence samples that (1) estimates on the number of contributors in a mixture based upon flow cytometry histogram profiles, (2) estimates the human-specific DNA content in the sample based upon fluorescent signal intensities, and (3) differentiates cell populations in the mixture based contributor-specific attributes. The primary advantage of using this approach with our novel signatures is that all aspects of the proposed workflow are inherently non-destructive which is ideal for touch evidence samples since these are typically compromised and low in template quantity. The aims and scope of this project specifically address three operational requirements identified by the 2019 Forensic Technology Working Group: (1) Biological evidence screening tools that can address number and proportion of contributors, (2) ability to differentiate and selectively analyze DNA and/or cells from multiple donors or multiple tissue/cell types contributing to mixtures, with minimal or no sample loss, and (3) comprehensive, systematic, well-controlled studies that provide both foundational knowledge and practical data about "touch evidence" DNA transfer and persistence in the real world.

Project Design and Methods

The goal of Phase I was to (1) conduct a survey of novel molecular signatures across individuals and (2) use the observed variation to develop a workflow for rapidly identifying and quantifying contributor cell populations within touch/trace cell mixtures. Our initial strategy was to use fluorescently labelled antibody probes to survey the differences in the presence and/or abundance of two types of intracellular targets: hormone molecules and cytokeratin proteins. The distribution of fluorescence intensity across a cell population (i.e., fluorescence histogram) was then compared across contributor samples to determine whether this signature could be used to detect and quantify the number of individuals represented in a mixture sample. To accomplish this, single contributor epithelial cell samples were collected from ~80 individuals and allowed to age anywhere from 1 to 14 days prior probe hybridization and flow cytometry analysis. The goal of Phase II was to test whether these signatures identified in Phase I were correlated to amount of recoverable DNA and/or the quality of DNA profiles from touch/trace samples. As a final task we tested whether signatures could be coupled to cell separation (via FACS) to create single-source DNA profiles from mixture samples.

Data Analysis

For all experiments, cell population samples were comprised of either epidermal cells or buccal/saliva epithelial cells. Each cell sample was deposited into a non-porous substrate (e.g., microscope slide, benchtop), allowed to dry, and then incubated at room temperature for between one day and two weeks. Epithelial cell deposits were collected from each surface using a pre-wetted cotton swab. Cell populations were then eluted from the swab using water, subjected to probe hybridization and then analyzed by flow cytometry using previously developed protocols

(Miller et al., 2022; citation below). To ensure that methods developed in this project are compatible with operational workflows of DNA caseworking units, all tasks involving DNA profiling of hybridized and/or sorted cell populations were executed using validated caseworking protocols for DNA profiling and interpretation at VA-DFS (Richmond, VA).

Findings

Survey of hormone signatures for differentiation of contributor cell populations

For this set of experiments, a series of 'touch' cell samples were generated by taking direct swabs of an individual's palmar surface or by having participants handle various substrates (e.g., plastic tube, knife handle, microscope slide). A total of 50 cell contributor populations were analyzed. Replicate samples from the same contributor were also collected to assess intra-donor variability. Overall, results showed that some contributor cell populations showed higher binding affinities for testosterone-probes as assessed by comparing the median fluorescence intensity for the entire cell population as well as the mode fluorescence intensity which corresponded to the peak in each fluorescence histogram. Among the contributors surveyed, approximately 30% showed differences in median and mode fluorescence intensity for respective cell populations. However, a large proportion of the contributor combinations exhibited no discernable differences in probe binding efficiency.

To further characterize the interactions between testosterone probes and individual epithelial cells, hybridized cells were also analyzed using confocal laser scanning microscopy. Results showed that testosterone labelled cells exhibited stronger fluorescence compared to both unstained and isotoype controls indicating some degree of molecular specificity with this assay.

Additionally, we observed significant heterogeneity in cellular fluorescence across cells, consistent with the biochemical and structural properties of shed epidermal cells.

Scientific results from this portion of project phase were disseminated at multiple scientific conferences including AAFS 2021 and 2022. Additionally, the above results were included in the following publication:

Miller, J., Lee, C. Ingram, S. Yadavalli, V.K., Greenspoon, S.A. **Ehrhardt, C.J.** 2022. Use of hormone-specific antibody probes for differential labelling of contributor cell populations in trace DNA mixtures. Int. J. Legal Medicine. 136(6): 1551-1564. doi: 10.1007/s00414-022-02887-x

Survey of cytokeratin signatures for differentiation of contributor epithelial cell populations

As part of Phase I, we also tested the binding efficiency and differentiation potential of antibody probes targeting structural alleles of cytokeratin molecules (specifically, probes AE1 which is specific to acidic keratin molecules and AE3 which targets basic keratin molecules). A total of 30 cell contributor populations were analyzed. Replicate samples from the same contributor were also collected to assess intra-donor variability. Due to differences between CK targets and hormone targets within the cell, initial effort focused on optimizing incubation conditions for CK probe binding such that non-specific binding with isotype control probes was minimized. We found that extending the incubation time (e.g., overnight incubation compared to 1 hour), using higher concentration of blocking buffer to reduce non-specific binding, and standardizing the ratio of antibody probe to cell concentration, effectively decreased non-specific binding as indicated by differences in median fluorescence between the isotype control and AE1/AE3 hybridized cell populations. Following these protocol adjustments results showed that certain contributor cell populations showed different affinities for cytokeratin probes AE1 and AE3. The most pronounced differences were observed with AE3 probe that targets basic cytokeratin structures. However, the differences observed in fluorescence between contributor cell

populations after probe hybridization were considerably less compared to cell populations after hybridization with testosterone probe (even between the same pairs of contributors). Interestingly, systematic changes in binding efficiency were also observed in cell populations from the same contributor but with different time-since-depositions. This is likely driven by increases in autofluorescence due to cellular degradation. Because of this, cytokeratin probes may not be an effective tool for detecting the presence of multiple contributors within an unknown DNA sample

Correlation between antibody binding and DNA content in trace biological samples

In Phase II we tested the correlation between antibody binding efficiency (both testosterone-specific and cytokeratin-specific probes) and the quantity of amplifiable DNA in a trace biological sample. In one experiment involving 20 contributor cell populations, there was some evidence of a linear relationship/correlation between the magnitude of cellular fluorescence and, therefore, affinity for testosterone probe and intracellular DNA yield. Specifically, approximately 66% of the variation in DNA yield between samples can be explained by variation in cellular fluorescence (R^2). This equated to a standard error of the estimate (SEE) of 0.15ng DNA which is the average error if this linear relationship were used to predict the quantity of amplifiable DNA based on cell population fluorescence following testosterone probe binding.

In contrast, results from parallel experiments using AE3 probe showed no evidence for a linear relationship/correlation between DNA yield and probe binding efficacy. For 15 contributors tested, the explained variance (i.e., R^2 for the linear regression) was 0.001). However, as part of the antibody probe experiments, we observed a strong correlation between autofluorescence of an unstained cell population (used as a negative control) and time-since-deposition. When DNA yield and DNA degradation index were quantified concurrently with cellular autofluorescence profiles

(via Powerplex Fusion STR amplification), we observed a strong correlation (R^2>0.9) between median autofluorescence and DNA degradation for both individual contributor cell populations as well as combined data from multiple contributors (Figure 4), suggesting that autofluorescence intensity could be an effective screening or predictive tool for STR profile quality.

Testing probe labelling for front end separation of touch/trace epithelial cell mixtures

Since there was strong evidence for contributor specific variation in the binding efficacy of testosterone-specific probes, we tested whether front-end hybridization could facilitate a mixture separation workflow. This was tested on four different two-person mixtures that were composed of one male and one female contributor. The contributor cell ratio was assessed by analyzing each donor cell solution separately prior to processing the mixture sample. For Mixture 1, the cell ratio for contributors was ~1.5:1. Epithelial cell sin each mixture were physically sorted into one of three fractions: 'left', 'middle', and 'right' based on their respective fluorescence intensities following probe hybridization. Based on signatures determined from Phase I, we expected one contributor profile to be enriched in the right fraction (i.e., high fluorescence from probe binding), while the other contributor profile was would be enriched in the left fractions (low fluorescence from limited or no probe binding. The middle fraction should represent a mixture of both contributor cell populations. Results from sorting the first mixture showed that each sorted fraction contained alleles consistent with each of the two contributors. Next, we performed a quantitative assessment of cell population enrichment in each sorted cell fraction using TrueAllele® Casework (TA) analysis. Results from the unsorted mixture showed statistical support for both contributors. The Contributor 1 donor profile had a log(LR) of 8.9791, while the Contributor 2 donor profile had a log(LR) of 1.0887 in the left sorted fraction. The analysis of the

sorted middle fraction showed high log(LR) value for Contributor 1 (12.2969 indicating strong statistical significance supporting this contributor in the profile. On the other hand, a negative log(LR) value was produced for Contributor 2 indicating no statistical support for association with this fraction. Results from the right fraction showed evidence of both contributor profiles (log(LR)s of 4.1185 and 3.7525, respectively).

Results from the sorting the second mixture (contributor ratio ~1:1) showed that the middle fraction had more male contributor alleles, and at higher peak heights compared to alleles from the female contributor. Conversely, the right fraction showed more alleles from Contributor 2 compared to Contributor 1 in the unsorted mixture and the sorted middle fraction. Quantitative analysis with TA also indicated enrichment of each contributor's profiles in the middle and right fractions. The log(LR) for Contributor 1 in the middle fraction was 19.5523. The unsorted mixture indicated no statistical support for Contributor 1, consistent with the minimal number of unique alleles observed and excessive allelic and locus drop-out. The drastic increase in log(LR) value after sorting is strong evidence for selective enrichment of Contributor 1 epidermal cells in this fraction. The right fraction had a log(LR) of 2.9306 for Contributor 2, indicating some statistical support for Contributor 2 in this sorted fraction.

As a preliminary test for this cell separation workflow on unknown mixture samples, we created two additional mixtures composed of two individuals. However, unlike the previous two mixtures, single source cell populations from each donor were not analyzed prior to sorting in order to guide placement of the sorting gate. A single sorting gate was used to collect two fractions ('post-left' and 'post-right'). Quantitative analysis of the unsorted mixture samples showed strong support for both contributor profile; however, the contributor ratio was estimated by probabilistic modeling to be ~9:1. For Mixture 3, there was evidence that the Contributor 1 profile was highly

enriched in both the right and left fractions, log(LR) 24.537 and 29.0711, respectively (Table S2). However, there was little support for the Contributor 2 profile in either fraction, log(LR) <0 and 0.6731 in the right and left fractions, respectively. For Mixture 4, there was strong statistical support for each contributor in both the right and left sorted cell fractions. Differences were observed in the magnitude of the log (LR) values between the sorted cell fractions with Contributor 1 displaying a higher log(LR) association than Contributor 2 in the right fraction the opposite trend in the left fraction (12.263 vs. 6.9911).

Overall, results from Phase II sorting experiments indicated that coupling hormone-specific antibody probes with fluorescence activated cell sorting can facilitate enrichment for contributor cell populations for two-person mixtures. When applied to two additional mixtures in a blind fashion, evidence for successful enrichment was observed in one of the two, albeit not as pronounced as was observed in the first two mixtures presented. This indicates that sorting efficiency at this point in the development of this procedure may depend partly on the nature of the donor cell populations present. Results from sorting experiments were also disseminated in the following publication, Miller et al., 2022 (full citation above).

Cellular fluorescence signatures for differenting contributor epithelial cell populations

One of the primary scientific findings in Phase I was that antibody probe binding could differentiate some, but not all pairs of contributors. As an orthogonal strategy we also tested whether that cellular autofluorescence signatures of epidermal cell populations can differ across contributors and over time as the sample ages/degrades, since differences were initially observed in unstained control cell populations during Phase I experiments. Autofluorescence profiles of trace epithelial cell populations were characterized from ~50 separate contributors to investigate

the extent to which differentiation may be possible. Results suggested that some cell populations can be distinguished based on their autofluorescence signatures detected with flow cytometry based on their clustering within three to four different multivariate clusters. This indicates that there may be a discrete number of cellular phenotypes based on autofluorescence profiles that may be used to detect the presence of multiple cell populations and/or presumptively identify touch samples that may originate from a single contributor (analogous to phenotypes associated with blood typing). The primary dataset and discussion of its potential application were published in the following article:

DeCorte A, Wolfe G, Dailey N *et al.* Morphological and Autofluorescence Dataset for 'Touch' Epidermal Cell Populations Collected with Imaging Flow Cytometry. *F1000Research* 2024, **13**:1177 https://doi.org/10.12688/f1000research.156869.1

Integration of fluorescence signatures with other standard/ and/or low-cost microscopic platforms

As part of Phase II, we developed a novel, rapid workflow for characterizing cellular fluorescence using ultra-low cost microscopic platforms (e.g., iolight portable microscope, ~$800). As part of the workflow we also incorporated open-source image analysis tools to extract fluorescence data and analyze fluorescnce signatures from either individual cells and cell populations (e.g., 'Segment Anything' tool by Meta). Results showed that touch epithelial cells can be imaged directly using this microscope such that both morphology and fluorescence data is captured. Expectedly, the number of cells that can be analyzed using this platform in a given amount of time is lower compared to flow cytometry. However, the cost of the microscopic platform and its and ease of use may be amenable to rapid screening of evidentiary samples for collections of cells that have disparate fluorescence levels, suggesting the presence of multiple contributor cell populations.

Data generated from this project is available through the following doi-repository links:

https://doi.org/10.6084/m9.figshare.11354114.v1

https://doi.org/10.6084/m9.figshare.27068128.v3

Implications for Criminal Justice Policy and Practice in the United States

To simplify complicated DNA interpretation methodologies and 'blind' processing of evidence, there is great need for techniques that can rapidly and non-destructively screen samples/stains for mixed cell populations and estimate which are likely to provide probative genetic material. Scientific results from this project include a novel set of contributor-specific signatures that have the potential to circumvent many of these issues by utilizing novel intracellular targets to selectively tag cell populations. Because all aspects of this workflow are inherently non-destructive these results can be instrumental in bridging the gap between the demonstrated utility of fluorescence-based cell labelling and its relevance for forensic casework. As such the products of this research has the potential to increase the probative value of many types of mixture samples and reduce caseworking bottlenecks associated with complicated interpretation protocols for DNA mixtures.