Final Summary Overview (Award #2015-DN-BX-K024)

Front End Separation of Compromised Blood Mixtures for Single Source DNA Profiling

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Purpose

The primary objective of this project is to test new methods to increase the efficiency of antibody tagging and cell sorting on degraded blood samples with the ultimate goal of developing a robust approach for obtaining single source STR profiles from compromised biological mixtures. To accomplish this, the research plan involves first characterizing the biochemical effects of cellular degradation on probe binding on white blood cells and then tests specific molecular strategies to maximize cell recovery and the efficiency of both probe hybridization and cell sorting on samples with varying levels of degradation, varying contributor ratios, and varying numbers of starting cells. To increase the impact of this research for forensic casework, we evaluated the effectiveness of these same techniques on other types of compromised mixtures including blood-epithelial and blood-buccal. This aims and scope of this project specifically address two operational requirements identified by the 2014 Forensic Technology Working Group: (1) Ability to differentiate, physically separate, and selectively analyze DNA and/or cells from multiple donors or multiple tissue/cell types contributing to mixtures, and (2) Ability to differentiate and tag a cell, identify and associate the biological source through to profile generation.

Project Design and Methods

The goal of Phase I was to characterize the effects of cellular degradation on HLA-antibody hybridization efficiency and test molecular strategies for maximizing differentiation of individual

cell populations in a mixture based on their HLA type. Anonymized blood samples were acquired from the Tissue Data Acquisition and Analysis Core (TDAAC) at the VCU School of Medicine. The HLA profile of each sample was determined by hybridizing fresh (i.e., liquid) blood samples to allele-specific antibody probes and assessing probe binding efficiency using flow cytometry. For these experiments, we primarily focused on the antigen products of two different HLA alleles, each from one of the three major Class I subgroups: A*02 and B*08. These alleles represent a wide range in terms of prevalence (possessed by ~45% and 20% of individuals, respectively).

The goal of Phase II was to test HLA-antibody assays and FACS for dried blood mixtures that approximate forensic caseworking samples. This includes samples with varying contributor ratios and limited number of cells. Additionally, we conducted a pilot study in Phase II to examine whether protocols developed in Phase I can be applied to forensic mixtures containing cells originating from tissue sources other than blood (e.g. saliva, epidermal). This pilot study included an investigation into an optimal set of HLA probes to maximize chances of fully separating an unknown mixture into individual contributors.

Data Analysis

Antibody hybridization was assessed by comparing fluorescence histogram distributions for target and non-target cell populations and extracting absolute cell counts at different relative fluorescence intensity levels. The effectiveness of the overall workflow (probe labelling and cell sorting) on resolving contributor cell populations from the mixture was determined by comparing STR profiles of two sorted cell fraction to each other and against the STR profile generated for unsorted mixture. The degree of enrichment/separation of component cell populations was

established by comparing allelic heights of the target and non-target contributor in each sorted cell fraction.

Findings

White blood cell characterization and antibody hybridization survey in dried samples

White blood cell populations from 35 different anonymized blood donor samples were dried for between 12 and 120 hours and hybridized with the HLA-A*02 antibody probe. The percentage of recoverable, intact, and antigen presenting white blood cells varies between ~80% and 95% after drying whole blood samples for at least 12 hours. However, the number of intact white blood cells decreases significantly after 24 hours, with no antigen presenting white blood cells resolved against background after three days (~80 hours). We note that although there is a significant decrease in antibody probe targets after drying, we have developed antibody labelling and flow cytometry protocols that allowed us to routinely capture hundreds to thousands of white blood cells from most of our donor samples. Specifically, for blood samples dried for less than 48 hours, probe concentrations of $0.5ug/10^3$ cells exhibited the greatest binding efficiency to target cells and the least amount of non-specific binding to non-target cell populations. Additionally, we have identified two different cell subpopulations based on forward scatter and side scatter profiles regions that show allele specific interactions with HLA antibody probe. One subpopulation corresponds to cells with larger sizes likely representing granulocytes, macrophages, and/or clusters of smaller white blood cell types. The second region exhibits smaller FSC and SSC values (correlated with cell size) and could represent other antigen-presenting white blood cells (lymphocytes) or degraded states of larger white blood cells.

Separation of two-person dried blood mixtures

As part of our initial assessment of the efficacy of antibody hybridization and cell sorting of dried blood mixtures, we created seven different two-person mixtures. Each mixture consisted of one contributor that was positive for the HLA-A*02 allele and one contributor that was negative for the same allele. Blood from each contributors was added to the mixture in equal volumes prior to drying. Mixture volumes ranged from 50µl-500µl and were dried for approximately 24 hours. Flow cytometry analysis of dried cell populations after antibody hybridization showed two distinct peaks, corresponding to each contributor cell population. We were able to use the differences in fluorescence intensity between contributor cell populations to define sorting criteria for isolating each mixture fraction. STR results from all seven mixtures showed successful isolation of both A*02 positive and A*02 negative contributors. In some instances, we observed minor contributions from the non-target cell populations. However, allelic ratios of the target:non-target contributor ranged from 10:1 to 75:1, allowing for easy determination of single source STR profiles from each fraction. These results were included in the peer-reviewed publication:

Stokes et al., 2018; Simplification of complex DNA profiles using front end cell separation and probabilistic modeling; Forensic Science International-Genetics 36: 205-212. doi: 10.1016/j.fsigen.2018.07.004.

Next, we completed follow up experiments with two person dried blood mixtures with varying contributor ratios: 10:1, 5:1, 3:1, 1:1, 1:3, 1:5, and 1:10. Mixture volumes ranged from 400 µl to 600 µl and dried for 24-48 hours prior to antibody hybridization and flow cytometry. Results showed that the profile of the minor contributor was enriched in each sorted cell fraction when compared to the STR profile of the unsorted mixture (profile of the major contributor possessed roughly equivalent allelic ratios in the sorted cell fraction compared to the original cell mixture). For example in the 1:10, 1:5, and 1:3 mixtures, the ratio of minor contributor increased

to ~1:2, ~1:1, and ~1:1 respectively. There was no systematic relationship between the degree of profile enrichment and the original contributor ratio. However, we did observe that the position of the sorting gate had a significant effect on the quality of the resulting profile, such that gates designed to capture cells at the tail ends of the fluorescence distribution of the mixture showed the highest enrichment for the target cell population. To test this, we created three different dried blood mixtures with 100 µl total volume and 1:1 contributor ratio. We then sorted the tails of the fluorescence distribution in a blind fashion (i.e., without measuring the fluorescence histograms of single source cell populations). The resulting sorted cell fractions showed enrichments of 2:1 and 3:1 for the A*02 positive and A*02 negative contributor respectively. This was consistent across two mixture replicates. The third blind sorted mixture contained two A*02 positive contributors; the resulting sorts of distribution tails showed 1:1 allelic ratios.

In general we noted that the overall fluorescence profile of the mixture shifted depending on whether the A*02 positive or negative contributor was dominant, i.e., when A*02 positive cells were the major contributor the overall histogram distribution was shifted to more intense fluorescence values and vice versa. This may be explained by physical aggregation of cells from the two contributors in the mixture during drying and re-elution prior to flow cytometry.

We also conducted a limit of detection test for cell sorting workflow by processing mixtures with 100 µl, 50 µl, and 20 µl total sample volumes (1:1 contributor ratio for each). Results showed enrichments of the target population of 2:1 to 23:1 suggesting that clear enrichments can be obtained for blood mixtures that originally contained as little as 20 µl of total volume.

Separation of two-person blood-buccal cell mixtures

We tested the hypothesis that buccal cells could be differentiated and sorted on the basis of their red autofluorescence intensity to produce single source STR profiles of each contributor. Initially we tested a 1:1 blood buccal mixture that had been dried for 48 hours. Mixture histograms show two distinct cell populations based on red autofluorescence that are consistent with profiles from each contributor/cell type. Two cell populations were then sorted according to gates defined by the reference histograms. STR profiles from the left sorted fraction were consistent with blood cell contributor with no unique alleles from the buccal cell contributor detected. Major alleles in the STR profile generated from the right sorted fraction were consistent with buccal cell contributor. Alleles consistent with the blood donor were detected in the right fraction but in peak height ratios of 1:10 to alleles from the buccal cell donor.

Next we tested a blood-buccal mixtures with varying contributor ratios (10:1, 1:1; 1:10) that had also been dried for 48 hours. Fluorescence histograms showed distinct cell populations in 1:1 and 10:1 (buccal:blood) mixture samples. Sorting results from the 1:1 mixtures showed excellent enrichment of the blood contributor in the left fraction (allelic ratio of blood to buccal contributor was 7:1). The dominant alleles in the right sorted fraction were consistent with the buccal cell contributor. A few alleles from the blood contributor were detected near the analytical threshold. The left fraction of the 1:10 mixture (buccal:blood) produced a near single source profile consistent with the blood contributor. The right fraction of the 1:10 mixture was enriched for the buccal contributor at an allelic peak ratio of 2:1 (buccal was a minor contributor in the reference profile of the original mixture). The left sorted fraction of the 10:1 mixture was enriched in the blood contributor's alleles 2:1 compared to the buccal contributor. The right sorted fraction

showed excellent enrichment for the buccal cell contributor (~10:1). Only three alleles from the blood contributor were detected in this fraction.

The third buccal-blood mixture system tested was dried for one week and aliquoted into different contributor ratios: 10:1, 1:1, 1:10 (buccal:blood). Distinct cell populations were only evident in the 10:1 mixture histogram. For the 1:1 mixture, alleles in the left sorted population were enriched in the blood cell contributor 6:1 compared to buccal contributor alleles. The right sorted fraction was also enriched in the blood cell contributor 3:1 (which was the non-target cell). For the 1:10 cell mixture, the left fraction was produced single source profile consistent with the blood cell contributor. The right fraction was also dominated by the blood contributor profiles (10:1 peak height ratio relative to buccal cell alleles). The 10:1 mixture showed little to no enrichment for either contributor in their respective sorted fractions compared to the original mixture profile.

Autofluorescence profiling of forensically relevant cell types

One of the basic research goals of this project is to understand the factors contributing to cellular autofluorescence in dried/compromised biological samples, as it can contribute to inclusion of non-target cells during mixture sorting. As part of this effort, we have used flow cytometry to characterize autofluorescence of white blood cells at several wavelengths (~350nm-700nm) as a function of drying time ranging between 3 and 128 hours. One of the specific analytical tools we have used for this is Imaging Flow Cytometry (IFC), whereby each cell is interrogated by series of excitation lasers and, at the same time, imaged microscopically in both brightfield and fluorescence wavelengths simultaneously. This allows for autofluorescence to be characterized in relation to the structure and morphology of the cell. Additionally, images can be

used to make a series of cellular measurements including area, aspect ratio, length width, fluorescence intensity, fluorescence heterogeneity, and contrast. Importantly, as with most flow cytometry approaches, this technique is non-destructive and high-throughput—thousands of individual cell images are collected in seconds. Cells can also be analyzed directly with minimal preparative steps or laboratory manipulations.

We have used IFC to test whether autoflourescence can be used to determine the time since deposition for blood cell populations. Blood samples from three different donors were used to create a time series that included drying times of 3hrs, 12hrs, 24hrs, 48hrs, 72hrs, 96hrs, 1 week, 2 weeks, 3 weeks, and 4 weeks. Each sample consisted of 100 µl of whole blood and were deposited on a range of substrates including tile, stainless steel, and plastic (i.e., three different time series for each donor). We then collected data on individual cells for 83 different morphological/fluorescence variables and used multivariate analysis to look for systematic differences across time points. Interestingly, we found that the median autofluorescence of cell populations systematically increases across time points between 24 hours and 1 week. A predictive multivariate analysis (linear discriminant analysis) showed that blood samples between these time intervals can be clearly differentiated from each other. In particular, cells that had been dried for less than 3 hours, 24 hours and greater than 48 hours can be separated based on IFC measurements. A preliminary nested multivariate analysis of cells dried for greater than 24 hours (i.e., excluding 'fresher' cell samples) showed stronger differentiation of 24 hour, 48 hour, 72 hour, and 96 hour cell populations. These trends were consistent across three different individuals. After cell populations had been dried for one week, systematic trends/changes in median autofluorescence over time were less clear (e.g., median autofluorescence would increase between weeks 1 and 2, decrease between weeks 2 and 3, then increase between weeks 3 and 4). Overall these results

suggest that autofluorescence measurements can potentially be used to differentiate cells that have been aged for 1 through 4 days or distinguish cells that have been aged for over 1 week from cells that were aged less than 1 week.

Data from our cell sorting experiments with blood-buccal cell mixtures indicated that autofluorescence profiles may differ as a function of tissue type. To test this, we combined autofluorescence data generated for this project from blood cell and buccal cells with previously generated data for contact epithelial cells and vaginal cells. We focused primarily on autofluorescence and morphological signatures collected with IFC. Cell populations representing each of the three tissue types were collected from 10 unique donors (a total of 30 donor-cell type combinations) and dried between 24 hours and approximately 8 weeks. Our results showed that buccal and epidermal cell populations could be differentiated with the highest accuracy (~94%). The lowest classification rate of individual donor cell populations in this comparison was 80% with the majority of cell populations exhibiting classification accuracy of 95% or higher. The vaginal-epidermal cell classifications showed comparable results with an overall classification accuracy of ~91%. Two individual cell populations in this scheme exhibited markedly lower success rates (63% and 32%). However, the remaining cell populations had classification accuracy >80% with the majority >95%. Less differentiation was observed between buccal and vaginal cells with an overall classification accuracy of 78%. Seven donor cell populations still showed accuracies greater than 95% and three donor cell populations were below 60% accuracy. Overall these results suggest that autofluorescence profiling combined with multivariate analyses may be used to extract tissue-specific signatures from aged biological samples in a high throughput and non-destructive manner. These results were published in a peer-reviewed manuscript:

Brocato et al., 2018; Rapid differentiation of epithelial cell types in aged biological samples using autofluorescence and morphological signatures. PLOS ONE. 13(5): 1-12. doi: 10.6084/m9.figshare.5847933.

Implications for Criminal Justice Policy and Practice in the United States

This research can impact on the forensic community by adapting a technique widely used in biomedical research to the particular demands of forensic casework. Cell mixtures are a widespread problem in forensic laboratories that often leads to inconclusive results and loss of evidence. To avoid complicated DNA interpretation methodologies, there is great need for techniques that physically separate cells from different contributors prior to DNA extraction. Since most samples encountered in forensic casework are compromised to some extent, the research that we have conducted will be instrumental in bridging the gap between the demonstrated utility of FACS and its relevance for forensic casework. Further, in advancing the state of understanding of the mechanics of degraded cells, not only will this research open up a fertile field for study of forensic applications of FACS, it will also provide valuable information that can be applied to improve results in other areas of DNA analysis such as determination of cell type and the 'age' of biological evidence..