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The physical separation and single source DNA profiling of individual mixture components by RNA *in situ* hybridization-based cell type identification

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I. Purpose

It is now a matter of routine for the forensic scientist to obtain the genetic profile of an individual from DNA recovered from a biological stain deposited at the crime scene. However, during the commission of a violent crime two or more people are present and may have the opportunity of depositing biological material at the crime scene or onto an individual or individuals, thus resulting in a mixture of genetic material. The analysis of DNA profiles comprising mixtures of DNA from two or more people can be a complex task, particularly when the number of contributors is not clear, DNA is present from three or more individuals, one or more of the components is present at a low template level, and/or the components are present in similar amounts such that the contribution of each individual to the mixture is difficult to resolve. A limited number of tools are available to operational forensic laboratories in order to aid in the interpretation or handling of mixtures, particularly for mixtures in which prior separation of mixture components by morphological differences (i.e. sperm and non-sperm cells in sexual assault evidence) is not possible or feasible.

Analysis of these mixture types relies on the use of non-physical methods (i.e. software solutions) to determine potential genotypes and minimize information loss. Despite attempts to provide consolidated guidelines for mixture interpretation, there is currently no single method that accommodates all the challenges of forensic casework samples. Even when statistical evaluation of a mixed sample is possible, the probative value of the evidence is often significantly reduced compared to a single source sample due to the statistical uncertainties of attributing specific genotypes to individual contributors unless continuous models are used.

The inability to clearly resolve individual contributors in admixed epithelial cell mixtures that are often found in sexual assault cases, and the need for the use of complex statistical interpretations, necessitates further research aimed at developing new methodologies to aid operational crime laboratories in the analysis of mixture samples. The purpose of this project was to develop a novel strategy for the de-convolution of admixed non-distinguishable epithelial cell mixtures using RNA *in situ* hybridization for cell type identification prior to DNA profiling. The identification of the cell type prior to analysis would permit a physical separation of cell type populations (or mixture components) resulting in single source DNA profiling as well as tissue source attribution (body fluid or tissue source of origin) for an individual DNA profile.

II. Project Design and Methods

The specific aims for the project were as follows:

Aim 1. Cell type identification by RNA *in situ* hybridization
Aim 1A. Development of body-fluid/tissue specific RNA probes for cell type identification
Aim 1B. Development of an optimized collection, lysis and amplification strategy

Aim 2. Physical separation of mixture components after RNA-FISH/CISH cell type identification for single source DNA profiling
A**im 3. Validation of developed RNA-FISH/CISH assays for de-convolution of admixed samples*)***
Aim 3A. Stability
Aim 3B. Casework

To perform the RNA *in situ* hybridization, several commercially available kits were evaluated including: 1) Stellaris probes (Biosearch Technologies), fluorescent detection; 2) RNAScope® (Advanced Cell Diagnostics), v2.0 and v2.5 kits using both colorimetric and fluorescent detection; 3) QuantiGene®ViewRNA™ Tissue (Affymetrix, now ThermoFisher Scientific), colorimetric detection; 4) QuantiGene®ViewRNA™ ISH Cell Assay kit (Affymetrix, now ThermoFisherScinetific), fluorescent detection and 5) QuantiGene®ViewRNA™ miRNA ISH Cell Assay kit (Affymetrix, now ThermoFisher Scientific), fluorescent detection. The most successful results were obtained using the QuantiGene®ViewRNA™ miRNA ISH Cell Assay kit.

The QuantiGene® ViewRNA™ miRNA ISH Cell Assay can detect two mRNA and one miRNA species in one multiplex assay with Type 1 probes detected using alkaline phosphatase and Fast Red and Type 4 and 6 probes detected using Alexa Fluor labels (Alexa Fluor 488 and 650 respectively). According to the manufacturer, this kit is more sensitive than the QuantiGene® ViewRNA™ ISH Cell Assay in that it can be used for detecting only mRNA. The probes using in the ViewRNA™ assays are a proprietary set designed by the company bioinformatics specialists. A target-specific probe set includes ~20 oligonucleotide pairs that hybridize to the target RNA.

The workflow for the QuantiGene®ViewRNA miRNA ISH Cell Assay consists of four steps: 1) Sample preparation, where the cells are fixed and permeabilized; 2) Target hybridization, where the target-specific probe sets hybridize to each target miRNA and mRNA;

3) Signal amplification, where the pre-amplifiers hybridize to their respective pair of bound probe sets. The amplifiers and then hybridized to the pre-amplifiers and then multiple label probe oligonucleotides (either conjugated to alkaline phosphatase or fluorescent Alexa Fluor) are hybridized to their corresponding amplifier molecules; 4) Detection using a fluorescent microscope.All the reagents come as ready-to-use reagents with the exception of dilution being required for the wash and storage buffer.

The QuantiGene® ViewRNA™ miRNA ISH Cell Assay required extensive optimization of all protocol parameters in order to obtain cell-specific probe binding. We have found that different annealing temperatures and component concentrations (proteases, probes, etc) are needed between cell types as well as amongst probes for the same cell type. Optimization of this protocol was very time consuming given the number of steps and reagents used in the process. The resulting protocol is a 3-day protocol that is labor intensive, particularly on days two and three. Slides are sometimes evaluated on day 4, if an anti-fade reagent is used which typically sits over night. For vaginal candidates, a 1:2000 protease dilution is used in addition to the 3 hour probe annealing step performed at 40^{o}C. For saliva candidates, we have found that a 1:2000 protease dilution will also likely work, but a 38^{o}C probe annealing step is required. Pre-amplified and amplifier steps are performed at 42.5^{o}C rather than the recommended 40^{o}C. The very small nuanced differences in the protocol appear to have a significant impact on the success of the assay. Overall it is a technically demanding procedure that requires an appropriate level of training and practice to be successful.

Our original goal was to have a duplex reaction with a target specific mRNA probe for both buccal and vaginal cells. We were unable to successfully use the Type 4 probes and therefore only a singleplex reaction could be performed. While not ideal, it is possible to have

separate samplings of the same cell suspension and evaluate them with the different singleplex reactions, thus still permitting a determination of which cell types were present and subsequent isolation of the different cell populations.

We evaluated numerous mRNA candidates for the identification of vaginal epithelial and buccal epithelial cells, as well as two housekeeping genes: vaginal – DEFB4, TYROBP, PACRGL, IL8, YIPF6, CYP2B7P1, FUT6, FAM83D, CYP2A6 and DKK4; buccal – UPP1, HTN3, STATH, PRH1, SMR3B, PRB1 and PRB3; housekeeping genes – UBC and B2M. We also evaluated one miRNA (miR-205) which was selected as a possible buccal cell candidate. While promising candidates for vaginal epithelial cells have been identified, the identification of buccal-cell specific candidates has been more challenging. We are still in the process of optimizing candidates for buccal epithelial cells, but a few potential candidates have been identified. The use of housekeeping genes was not possible as a duplex reaction was not successful.

We took two approaches to the collection and analysis of labeled epithelial cells: 1) laser capture micro-dissection (LCM) and 2) micro-manipulation. The LCM method requires the dispersal of the cell suspension onto a proprietary membrane and the targeted cells are then circumscribed by, and cut out, by a laser. The targeted cells are collected into tubes which can then be used directly in DNA isolation or amplification protocols. With micro-manipulation, a water soluble-adhesive is collected onto the end of a tungsten needle. The adhesive is then used to collect targeted cells from the substrate (e.g. WF Gel-Film® (Gel-Pak®)). The cells are then transferred directly into a 0.2mL PCR tube containing a lysis or amplification solution. While a majority of the project was focused on the development of the RNA ISH protocol, preliminary data shows that labeled cells can be collected and STR profiles recovered.

III. Findings

A. Vaginal epithelial cells

The following mRNA gene candidates were evaluated as potential vaginal specific epithelial cell biomarkers: DEFB4, TYROBP, PACRGL, IL8, YIPF6, CYP2B7P1, FUT6, FAM83D, CYP2A6 and DKK4. The first five gene candidates listed are novel candidates identified by ESR and were not evaluated by the UCF laboratory due to the observed lack of specificity. The latter five in this list are mRNAs that have been used in other body fluid identification assays developed by our laboratories with more data available on their specificity. CYP2B7P1 was tested in both laboratories. After extensive evaluation and parameter optimization, we have been able to demonstrate specificity for vaginal epithelial cells for CYP2B7P1, FUT6, CYP2A6 and DKK4. CYP2B7P1 and FUT6 were the top two candidates, with CYP2A6, DKK4 and FAM83D showing weaker, but still specific, expression in vaginal cells. CYP2A6 required a 1:2000 protease dilution in order to obtain successful results. It should be noted that while specificity was reproducible within a laboratory, it was not observed between laboratories. This could be due to small differences in protocols, sample preparations or reagents since the work was performed in different countries. The images below show the expression of the top two candidates in vaginal epithelial cells with corresponding little to no expression in buccal epithelial cells. Expression is indicated by the presence of a red ‘dot’ (Type I probes, Fast Red detection). Only a limited number of donors have been tested thus far (~1-3 donors) and therefore additional validation studies are needed to ensure that the same expression levels and specificity will be observed in additional donors.

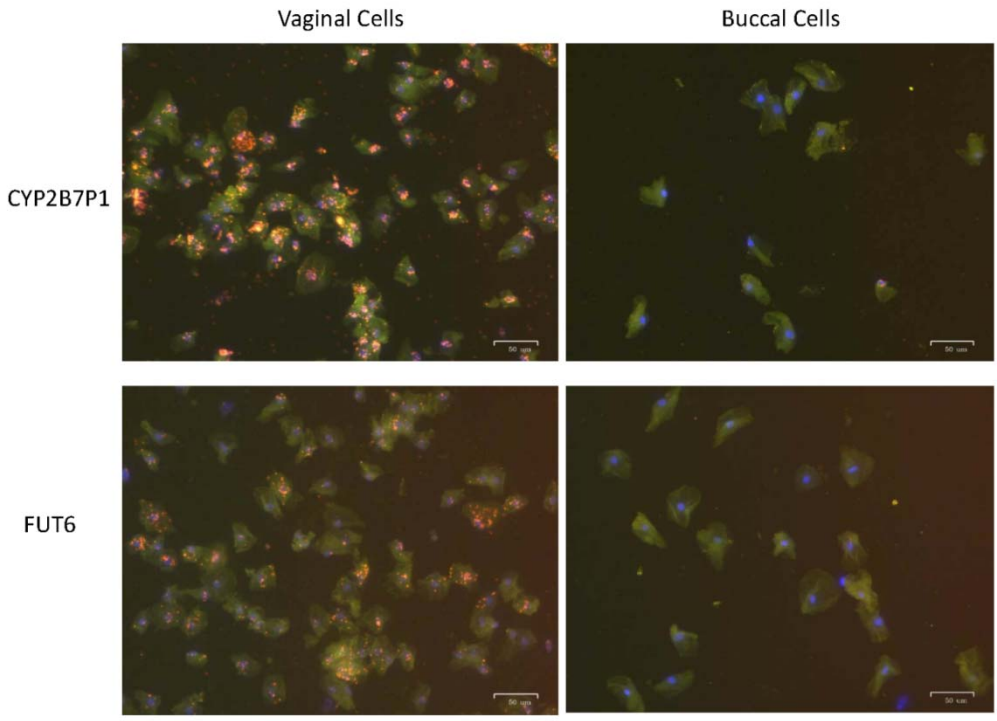

Figure 1. Expression of CYP2B7P1 and FUT6 in Vaginal Epithelial Cells Using RNA ISH

B. Buccal epithelial cells

The identification of suitable buccal cell specific probes was much more challenging. We evaluated UPP1, HTN3, STATH, PRH1, SMR3B, PRB1 and PRB3 as potential candidates with a majority of these genes previously identified and evaluated in other mRNA assays. The expression levels of the buccal cell candidates are much less compared to what was observed for vaginal secretions. However, recent results have indicated that the candidates may be demonstrating specificity for buccal cells (Figure 2, HTN3). Additional optimization work is required in order to attempt to improve signal intensity as expression is very weak in buccal cells and is not present in all donors. We will continue to also evaluate and optimize the other buccal cell candidates to try to improve sensitivity and specificity.

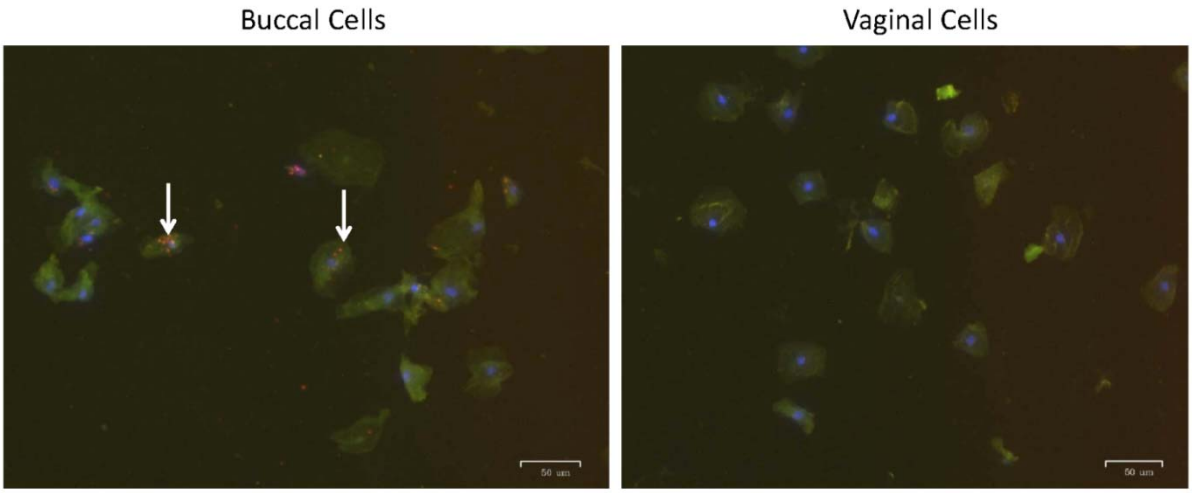

Figure 2. Expression of HTN3 in Buccal Epithelial Cells Using RNA ISH

C. No probe controls

To ensure that the observed expression signal is due to genuine expression of the mRNA in the cell, no probe (negative) controls are included with each experiment. These samples undergo the full protocol with the exception of no mRNA probe being added. An example of a 'no-probe' negative control is shown below. As can be seen, no expression is observed providing support that the observed signal is due to genuine mRNA expression.

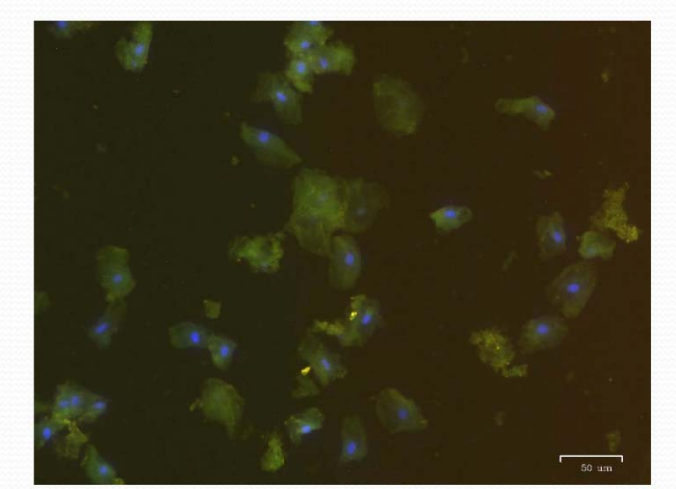

Figure 3. Example of a No-Probe Negative Control

D. Physical separation and DNA profiling

A main goal of the current work was to identify different epithelial cell types within an admixed sample in order to permit isolation ofbuccal and vaginal epithelial cells into different sub-samples for single source DNA profiling. While we do not currently have a complete assay in terms of specificity, we wanted to perform initial DNA profiling experiments to determine if the ISH probes would interfere with or inhibit subsequent STR amplification reactions.

We took two approaches to the collection and analysis of labeled epithelial cells: 1) laser capture micro-dissection (LCM) and 2) micro-manipulation. LCM was evaluated using buccal cells labeled with miR-205 (Type I probe) and immobilized on PET (polyethylene terephthalate) membrane slides. A full Identifiler® Plus STR profile was obtained for a 150-cell sample (one-step DNA extraction method). Additional sensitivity studies using 100- and 150-cell samples, however, did not yield full profiles. Partial profiles were obtained for all samples with profile completeness values ranging from ~40 to 94% for most samples. The second approach involved the use of micro-manipulation. Since we did not want to introduce any potential background fluorescence from the Gel-Film®, we were able to collect cells directly from the glass slide that the cells were on. This was challenging due to the use of the anti-fade reagent. Future work will determine whether the expression signals can still be observed without the use of antifade reagents for at least a day or two after the viewRNA protocol is complete. This would be ideal as routine recovery of cells from the antifade reagent does not seem very feasible. We were able to perform testing on a STATH-labeled buccal cell sample, both with and without antifade added. With the antifade reagent, we obtained an almost full STR profile with only 5 buccal epithelial cells (two loci and one allele drop out). Without the antifade added, we collected 5- and 10-cell samples from two donors. For the 5-cell samples, 29/30 and 22/30 alleles were recovered in each

of the samples. For the 10-cell samples, 29/30 and 30/30 alleles were recovered in each of the samples. Therefore, this initial data suggests that STR profiling from the labelled cells by micro-manipulation will be possible.

E. Validation

Assay development has not reached the appropriate stage in order for formal validation experiments to be performed. Further work will be performed during the remaining performance period in order to possiblyimprove the robustness of the assay.

IV. Implications for criminal justice policy and practice in the United States

Interpretation of admixed STR profiles from multiple donors present a complex challenge for forensic laboratories. Few studies have been carried out to aid the scientific community in the physical de-convolution of non-distinguishable cell type mixtures. The current work sought to help the scientific community in this regard by developing a novel strategy to identify the epithelial cell type of origin (buccal, vaginal or skin) using novel RNA *in situ* hybridization techniques in order to permit physical separation or 'de-convolution' of undistinguishable epithelial cell mixtures prior to DNA profiling. This prior cell type identification would permit both the positive identification of the cell type by RNA expression profiling and the single source STR profiling of individual contributors in mixture samples.

It is not clear at this time if this approach will be robust enough for routine use in forensic casework. Additional work is needed to refine and validate this approach before a determination can be made. The initial results indicate that RNA ISH can be used to identify vaginal, and possibly buccal, cells from dried stains, thus identifying them prior to collection and DNA analysis. This approach may be more useful in specialized cases, but not necessarily routine use.