The author(s) shown below used Federal funding provided by the U.S. Department of Justice to prepare the following resource:

Document Title:	**Sex-Based Targeted Recovery of Cells in a Heterogeneous Mixture: Separating Male and Female Like-Cells**
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Document Number:	**306007**
Date Received:	**February 2023**
Award Number:	**2020-DQ-BX-0019**

Technical Summary Report

Project title: *Sex-Based Targeted Recovery of Cells in a Heterogeneous Mixture: Separating Male and Female Like-Cells*

Principal Investigator: Michael A Marciano, Ph.D.

National Institute of Justice Award #: *2020-DQ-BX-0019*

Project Period: January 1, 2020 – December 31, 2022

Award Amount: $134,297.00

i. ***Project Summary***

A. Goals and Objectives

The central aim of this project is to develop and optimize a method to identify and recover male cells from a mixture of male and female like-cells, e.g. epithelial cells. This is done by targeting (immunostaining) the male Y-chromosome with a chromosome paint followed by the use the DEPArray NxT to detect and recover male cells. We will combine two well characterized methods into a single unified protocol for use in forensic DNA analyses. There has been widespread use of the proposed technique for Y-chromosome identification, both within and outside of the forensic arena [1–6]. For example, Anslinger et al. 2007 was able to successfully and clearly distinguish male cells from female cells using the Vysis Abbott DNA Probe Kit [6]. Pairing this Y-chromosome labeling method with the DEPArray is critical to the success of this project, where the positively stained cells will be able to be selectively recovered. Phase I of this project sought to adapt and optimize male specific cell immunostaining using a commercially available Y-chromosome staining kit (phase I) prior to use on the DEPArray™ NxT. The method will be assessed using measures of accuracy, precision (true positives/ true positives + false positives) and sensitivity (true positives/ (true positives + false negatives)). Phase II focused optimizing the detection of the stained cells using the DEPArray™ NxT. This phase will also include sensitivity, specificity, and a mixture study. The final phase of this project evaluates the methods developed in the previous phases with non-pristine epithelial cells, including a sensitivity study and mixture study.

B. Research Questions

Primary research question: Is it possible to selectively label and recover male epithelial cells in a mixture with female epithelial cells?

Detailed research questions:

1. Can a commercially available method (Abbott Molecular Vysis CEP Y (DYZ1) probe) [7] to label human Y-chromosomes be modified to successfully stain cells in suspension?
2. Given the success of (1) can the method be transitioned to successfully detect and recover the male epithelial cells using the DEPArray NxT or DEPArray PLUS? Sub-studies include:
    a. *Sensitivity* – What is the labeling efficiency (true positive rate) of probe binding and detection using the modified protocol developed in (1)?
    b. *Specificity* – What is the false positive rate and true negative rate of the Y-chromosome labeling method developed in (1)?
    c. *Mixture Study* – Evaluate the performance of the method validated in the sensitivity/specificity study using human DNA mixtures consisting of varied ratios of female cells to male cells, simulating casework scenarios. This sample set will include three mixture samples with male to female buccal epithelial cell ratios of 1:1, 1:10 and 1:100.
3. Is the method for labeling and recovering the male cells described in (1) effective in labeling and recovering male cells in non-pristine samples (samples that are 10+ years old)?

C. Summary of Project Design and Methods

Project Design

i. Sample sets: (1) Cell line samples will be used to perform an initial evaluation of the cell straining procedures. Two cell lines will be used to test the robustness of identifying female from male cell populations. The female immortalized cell line is a cervical cancer cell line, HeLa cells,

and the male derived cell line is derived from the prostate epithelium (RWPE-1), (2) fresh buccal swabs collected from volunteers and (3) the Acadiana Crime Laboratory will provide a sample set comprised of pristine and aged/degraded buccal swabs that are between 10 and 20 years old. These samples will simulate true casework samples that may be encountered when the proposed methodology is needed. The sample set will be made from pre-existing laboratory buccal swab samples. Note, the degraded samples are >10 year-old buccal swabs left at room temperature and are used routinely by the Acadiana Laboratory to assess/validate new methods using degraded samples, such as those that may be encountered in casework.

ii. Phase I: Cell staining optimization. This phase evaluates, a commercially available Y-chromosome staining kit (Abbott Molecular Vysis CEP Y (DYZ1)). The evaluation will first be performed on slides (per manufacturer recommendations) and will be transitioned to in-suspension, prior to use on the DEPArray™ NxT. The method(s) will be assessed using measures of accuracy, precision (true positives/ true positives + false positives) and sensitivity (true positives/ (true positives + false negatives)). Our focus is to minimize the level of false negatives and staining will be deemed a success if the true positive rate is >90%. The methods are as follows: (1) *Method 1:* We will evaluate a commercially available method [7], modified to stain cells in suspension, to target and fluorescently label the human Y-chromosome (Abbott Molecular Vysis CEP Y (DYZ1)) probe. This will be performed in triplicate for male and female cell samples. The Y-specific probe to be used (conjugated to dyes – Spectrum Green or Spectrum Orange dyes) hybridizes to satellite III DNA at in region Yq12 [7]. This probe targets centromeric regions and will not interfere with any forensically relevant Y-STR or autosomal loci. We do not expect the probes, nor the blocking DNAs used in the kit, will cause nonspecific priming during PCR, as these are much larger than the typical 17-25 bp sizes of primers. We

expect this method to be successful in achieving a >90% true positive rate. This method, applied in bone marrow samples, was shown to yield a 100% true positive rate with no false positives. Again, this method has been shown to be highly sensitive and specific in the presence of mixtures, where, in bone marrow tissue, an average of 0.88% ± 0.48 of male cells were detected in 1:99 mixture of male to female cells and 4.9% ± 0.99% if males cells in a 1:19 male to female mixture [7]. Numerous independent research studies have also demonstrated success using the proposed method for Y-chromosome labeling [6-9]. Cells will be prepared and imaged on a spinning disk confocal microscope where signal to noise will be analyzed between the female cell line (unlabeled) compared to the male cell line. This microscope utilizes an 89 North – LDI 6-line laser launch that works with a Photometrics Prime-95B camera attached to a Crest Optics: X-light V2 Confocal Unit spinning disk.

iii. Phase II - Optimizing the detection of the cells using the DEPArray™ NxT. The optimized staining protocol identified in *phase I* will be incorporated into the DEPArray™ run preparation protocol used in [10]. The DEPArray NxT has the dye channels necessary to accommodate the detection of the dyes validated in Phase I (Table 1).

Table 1. Fluorescent probes and their respective DEPArray™ channels to utilize to differentiate between male and female epithelial cells[11-12].

Fluorescent Probe	Excitation Peak	DEPArray™ Channels	Excitation wavelength (nm)	Emission wavelength (nm)
SpectrumGreen™	497	FITC	469	510
SpectrumOrange™	559	PE	546	581
DAPI counterstain	367	DAPI	376	447

Phase II is comprised of four sub-studies evaluating Sensitivity, Specificity, mixture analyses and compromised samples. (1) *Sensitivity* and (2) *Specificity* – these sub-studies characterize the accuracy, sensitivity (true positive rate), specificity (true negative rate) and false positive rate of

the Y-chromosome staining method used in conjunction with the DEPArray™. This will be accomplished on two DEPArray runs, where male and female only samples are stained and added to each cartridge. Staining specificity can be quantitated by counting cells and sensitivity will be assessed by comparing the pre-DEPArray cell counts (from phase I analyses) with those observed in the active area of the cartridge. A female and male sample will be stained and run on the DEPArray separately, five recoveries will made attempting to maximize the number of labeled cells per recovery. It is expected that the female cell only DEPArray run will not yield any labeled cells. The male recoveries will be extracted, amplified and run on the 3500 Genetic Analyzer using the SU forensic DNA pipeline. The resulting data will be assessed using the procedure described in Methods section v. (3) *Mixture Study* – This sub study will evaluate the performance of the method validated in the sensitivity/specificity study using human DNA mixtures consisting of varied ratios of female cells to male cells, thus simulating casework scenarios. This sample set will include three mixture samples with male to female buccal epithelial cell ratios of 1:1, 1:10 and 1:100. Each mixture sample will have six (male) cell recoveries performed, each comprised of the maximum number of recoverable cells. The six recoveries from each mixture sample (18 total samples) will be split, half (9 samples) analyzed by the Acadiana Laboratory and half analyzed by the SU laboratory. Wet-bench and analytical pipelines are described in the Methods sections.

iv. Phase III - Non-pristine sample evaluation and optimization. Non-pristine samples are routinely encountered in casework and therefore this method must be validated using degraded DNA samples. This phase will evaluate the performance of the methods validated in Phases I and II on non-pristine samples. Degraded samples, prepared as described in the Methods section (i. Sample preparation), will be processed using the optimized methods in phase I and II. This

evaluation will consist of four degraded single source samples (three male and one female) and three degraded mixtures at ratios of 1:1, 1:10 and 1:100 (male:female). Six recoveries, consisting of the maximum number of cells possible, will be collected from each sample, yielding 36 total.

Methods

i. Sample preparation

This study evaluated the staining efficiency on three cell types: cultured cells, fresh buccal epithelial cells, and old buccal epithelial cells. Preparation of cultured cells, female Hela cells derived from ovarian cells and male RWPE-1 prostate cells, required resurrection from a stock. These cells are aliquoted into 60 mm tissue culturing plates containing eight 1.0 cm glass slides. The cells were incubated until they reached confluency, (i.e., when the cells have grown across the entirety of the glass slides). Upon confluency, the cells were fixed by adding 5.0mL of a 3:1 methanol: acetic acid to the glass slides dropwise. Fresh buccal epithelial cells were collected from swabs of the inner cheek of the male and female participants. Next, because of the *in-solution* requirement of DEPArray mediated detection and recovery, the cells were removed from the swab and suspended in Phosphate Buffered Saline (PBS). This was completed by addition of the swab into a lo-bind microcentrifuge tube with approximately 500 µL of PBS. The tube was gently vortexed and shaken at 300 rpm for 20 minutes to release the cells from the swab. The swab was then moved to a spin ease basket and centrifuged at 600 x g for 3 minutes. The swab and basket were removed and the fresh buccal epithelial cell stock, male or female, was resuspended. The same procedure was followed to create in-solution samples of aged buccal swabs. Mixtures of fresh or aged in-solution buccal epithelial cells were prepared from the in-solution buccal epithelial cell stocks. The stocks (male and female) were counted using a

hemocytometer and diluted or concentrated to contain 100 cells/µL. Then, the stocks were used to produce samples with male to female ratios of 1:1, 1:10, or 1:100 and approximately 10,000 cells. Old mixture samples were made to contain only 5,000 cells due to the limited number of total cells/swabs available.

ii. Y-Chromosome staining procedure and staining efficiency

The Abbott CEP Y Spectrum Green and Orange probes have been previously demonstrated to selectively label the Yq12 satellite III region of the Human Y chromosome on slides [7]. The manufacturer's recommended protocol was adapted and optimized for this application (Figure 1). Staining efficiency was evaluated first by testing the stain on cultured cells (male-RWPE prostate cells and female-HeLa cells) on a slide. When determining staining efficiency, a cell was identified by positive DAPI staining of the nucleus in the DAPI channel. Thus, the total cell count in a field of view was determined to be the number of DAPI positive cells observed. A positive male cell was identified by Y-probe staining within the nucleus in the FITC (green probe) or PE channel (orange probe). Approximately 10-15 fields of view were analyzed per sample. Upon staining efficiency evaluation on cultured cells, the staining method was evaluated in-solution on fresh buccal epithelial cells. The optimization of the method in-solution included testing the efficacy of detergents (Triton, NP-40), internally made denaturation solution and Abbott hybridization buffer, 3:1 methanol/acetic acid and 2% PFA, incubation times - 1 hr., 3 hr. and overnight. Approximately 5 to 25 µL of in-solution sample was added to a slide and the staining efficiency was evaluated with the same method as the cultured cells. Approximately 5 µL was used when the rest of the sample was intended to proceed to DEPArray analysis and up to 25 µL was used when only assessing staining efficiency. After optimization of the in-solution staining protocol and staining efficiency evaluation on fresh cells, the staining

method was evaluated in-solution on old buccal epithelial cells using the same procedure described for the fresh cells. The optimized staining procedure is as follows:

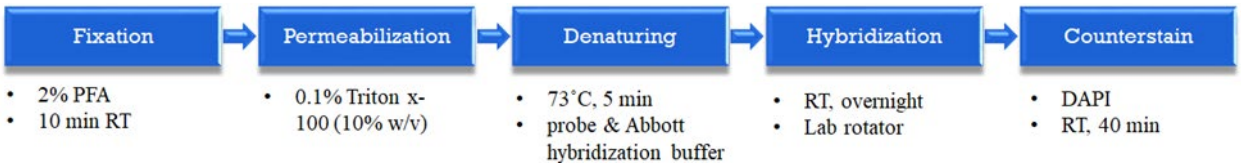

Figure 1: Overview of the cell staining process

iii. Visualization of Y-chromosome staining

Visualization was performed on a Zeiss LSM 980 Airyscan Confocal 2 microscope at 63x oil immersion, Olympus IX50 fluorescent microscope (400x) and the Zeiss Axioscope 5 fluorescent microscope (400x).

iv. Routing and recovery using the DEPArray NxT and PLUS

The DEPArray NxT and PLUS were used to identify and recover Y-probe-stained cells and DAPI stained cells (Figure 2). Manufacturer recommended protocols were used for sample preparation and instrument protocols, with the "forensic recovery" setting. The individual dye channel settings for DAPI and FITC or PE are largely based on the quality and intensity of the signal, generally the settings used were as follows, DAPI: exposure time – 300 ms, focus offset – 35-50 µm, camera gain – 3%, lamp Intensity – 25% and FITC or PE: : exposure time – 1000ms, focus offset – 35-50 µm, camera gain – 3%, lamp Intensity – 100%.

Approximately 2,000 cells were loaded into the DEPArray cartridge per run (input capacity of 3,000 cells). The DEPArray PLUS was used in conjunction with the NxT due to increased sensitivity and lower signal to noise. This generally improved the ability to identify positively stained male cells. Cells were routed and recovered in three primary groups (1) single cells – male or female and (2) groups of cells – Y-probe positive cells or Y-probe positive cells

and Y-probe negative (expected male and female cells in a single recovery) and (3) Y-probe negative cells (expected female cells).

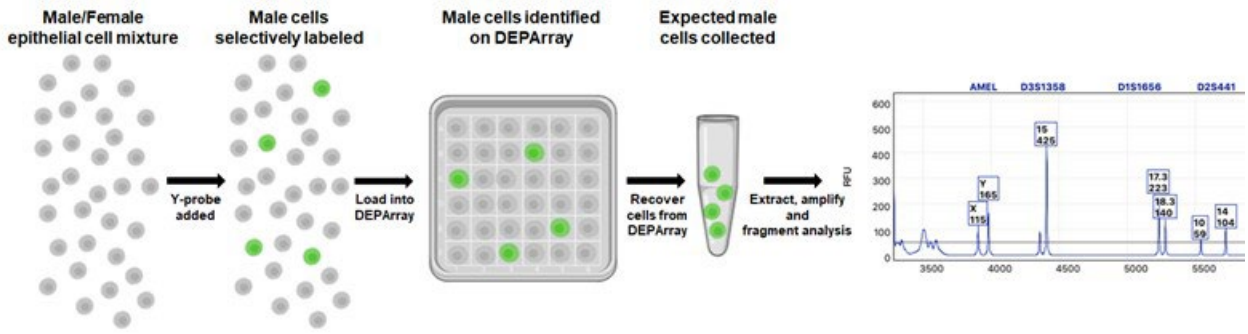

Figure 2: A diagram summarizing the basic steps in the cell staining and recovery process using the DEPArray NxT. Created with Biorender.com.

v. Analysis and interpretation

All samples were extracted using the DEPArray™ LysePrep Kit (Menarini Silicon Biosystems - DALYS) with 2 µL of master mix, and 1 µL of the DEPArray™ product. Amplification of the cell lysate (3 µL) using the of Promega PowerPlex Fusion 6C human DNA amplification kit (Promega DC2705) was performed by directly adding the amplification reagents to the extracted sample tube; half-volume reactions were used – 2.5 µL master mix, 2.5 µL primer pair, and 7.4 µL sample making the total volume 12.5 µL for 29 cycles. Fragment analysis was conducted using the Applied Biosystems 3500XL Genetic Analyzer (polymer - POP-4; capillary array – 36cm). Software analysis was carried out using Open Source Independent Review and Interpretation System (OSIRIS v 2.14). The 1:1, 1:10, and 1:100 male to female epithelial cell mixture recoveries were compared using the sample-wide mean peak heights at each locus and the proportion of alleles present in the sample. The mean peak heights calculations were made on alleles that were not shared between donors, and the proportion of alleles present included shared alleles.

D. Summary of Results

i. Optimization of the staining method

An initial study was conducted to demonstrate the effectiveness of the staining method using cultured RWPE (male) and HeLa (female) cells on a slide. The Y-probe binding and detection efficiency was observed consistently at or above 90% in the male RWPE cells and not observed in the female HeLa cells (Figure 3). Positive Y-probe efficiency was 92.24% ± 8.93% (214/232) on male RWPE cells with no staining observed (0/242) in the female HeLa cells (Table 2).

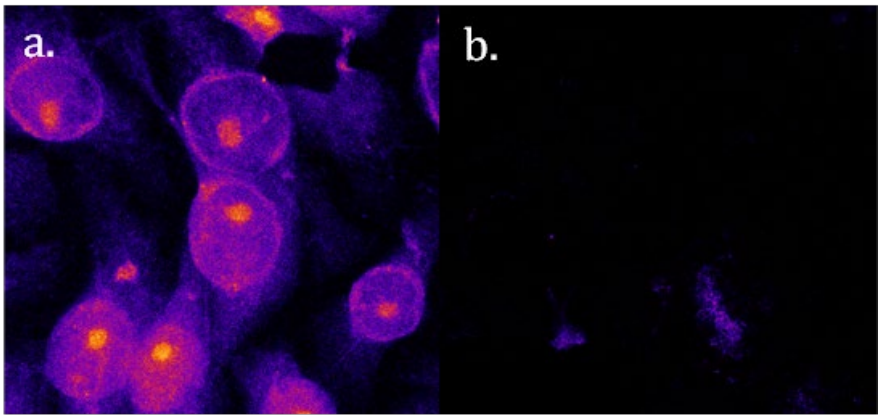

Figure 3: A single field of view on the LSM 980 Confocal microscope in the FITC channel of (a) RWPE cells (male) and (b) HeLa cells (female) prepared using a 0.2X probe concentration and incubated for 3 hours with the Spectrum Green Y probe and stained on a slide.

Table 2: The staining efficiency of the Spectrum Green and Orange probes with the RWPE male prostate cells, female HeLa cells and male and female buccal epithelial cells.

Probe color	Sample (cell type)	Staining method	# positively stained cells	Total count	Fields of view per stain (avg)	Number of Stains	Staining efficiency (%)
Green	RWPE	slide	214	232	5	1	92.24 ± 8.93
	HeLa		0	242	5	1	0.00 ± 0.00
Green	Male buccal	solution	295	429	2.67	3	68.76 ± 28.66
	Female buccal		1	207	3.67	3	0.48 ± 0.28
Orange	Male buccal	solution	215	289	10	2	75.41 ± 13.88
	Female buccal		0	224	10	1	0.00 ± 0.00

Upon successful staining of cultured cells on slides, male and female buccal epithelial cell staining was attempted with the green probe in-solution. Staining was completed in-solution due to the in-solution requirement of the DEPArray System. The in-solution staining method was optimized by varying the concentration of the probe, type and concertation of fixative, type of hybridization buffer, hybridization conditions (temperature and agitation - thermomixer and rotator) and number of post hybridization washes. A total of 4 samples were stained using the CEP Y Spectrum Green probe with varying hybridization times and concentration of paraformaldehyde (PFA) fixative. All samples were freshly collected male epithelial cells from buccal swabs stained using a 0.35x probe concentration: (1) 3-hour hybridization at room temperature with 1% PFA; (2) 3-hour hybridization at room temperature with 2% PFA; (3) overnight hour hybridization at room temperature with 1% PFA; and (4) overnight hour hybridization at room temperature with 2% PFA.

The Olympus microscopy imaging settings included a DAPI exposure of 625 milliseconds, FITC exposure of 2.5 seconds, an ISO sensitivity of 800 and high image quality. The cells were manually counted based on true positives and false negatives as described in the materials and methods section. A cell must be seen with the DAPI counterstain (DAPI channel) to be counted as a nucleated cell. Non-nucleated cells were not counted. The counting results can be found in Table 3. Data for the remaining optimization of parameters are not shown, the final procedure is shown in Figure 1.

Table 3: Cell counting results of initial validation of CEPY Spectrum Green Probe (in-solution), varying the concentration of fixative and hybridization incubation times.

Sample	DAPI positive	FITC positive	Probe binding efficiency (%)
3-hour 1% PFA	62	29	46.7%
3-hour 2% PFA	134	51	38.1%
overnight 1% PFA	161	133	82.6%
overnight 2% PFA	112	96	85.7%

Staining efficiency of the optimized green probe in-solution staining procedure (Figure 1) was evaluated and resulted in Y-probe signal in male cells (Figure 4 – a,b,c) and no Y-probe signal in female cells (Figure 4 – d,e,f) with staining efficiencies of 68.76% ± 28.66 and 0.48% ± 0.28%, respectively (Table 3, Figure 5 – a,b,c,d). Samples were prepared using the Spectrum Green probe and loaded onto the DEPArray NxT to evaluate the detection sensitivity of the DEPArray optics. The sign from the green probe (FITC channel) was generally low making it challenging to identify cells. Therefore the Spectrum Orange probe (PE channel) was evaluated and found to provide better quality signal, with a staining efficiency (in-solution, visualized on a slide) of 75.41% ± 13.88% and 0.48% ± 0.28% for male and female buccal epithelial cells, respectively (Table 3, Figure 5 – e, f, g, h). Thus, the remaining studies were completed with the orange Y-probe rather than the green Y-probe. Ultimately, the ability to detect male

cells using the DEPArray was confirmed, although at a lower rate than observed simply using the Zeiss Axioscope 5 fluorescent microscope (400x).

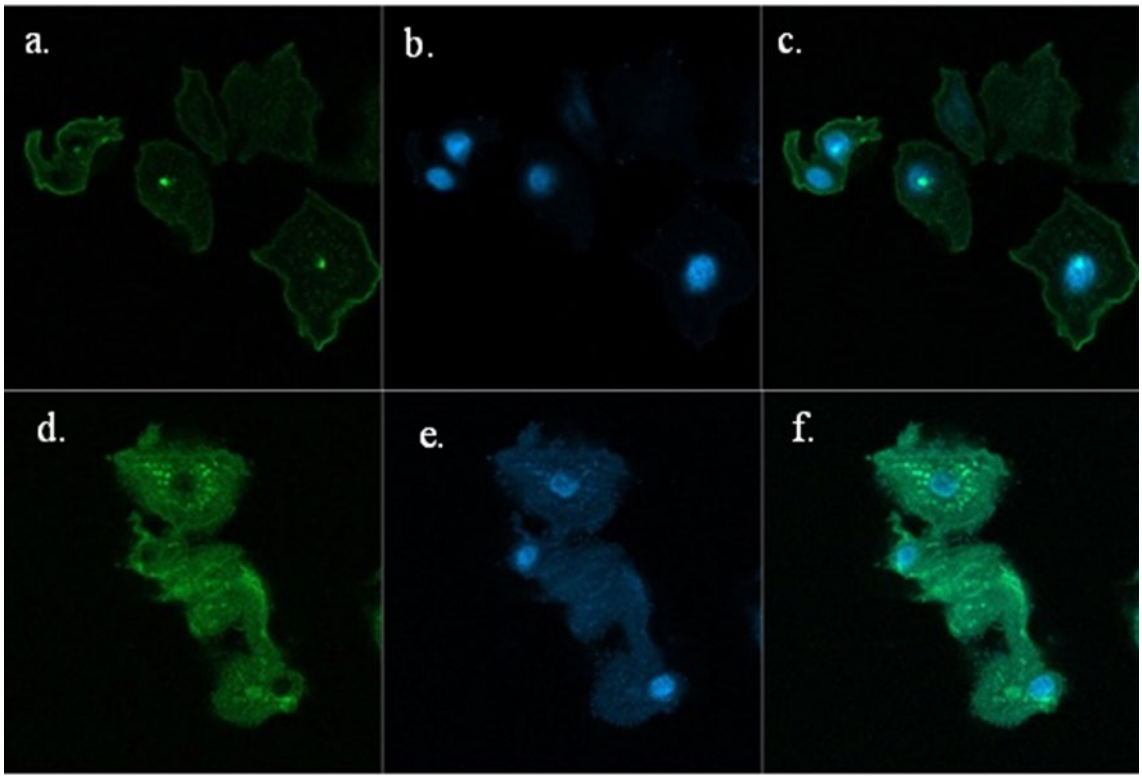

Figure 4: Male buccal epithelial cells stained with DAPI and the Spectrum Green Y-probe visualized in the FITC channel (a) and the DAPI channel (b) and overlay of both channels (c). Female buccal epithelial cells stained with DAPI and Spectrum Green Y-probe visualized in the FITC channel (d) and the DAPI channel (d) and overlay of both channels (f).

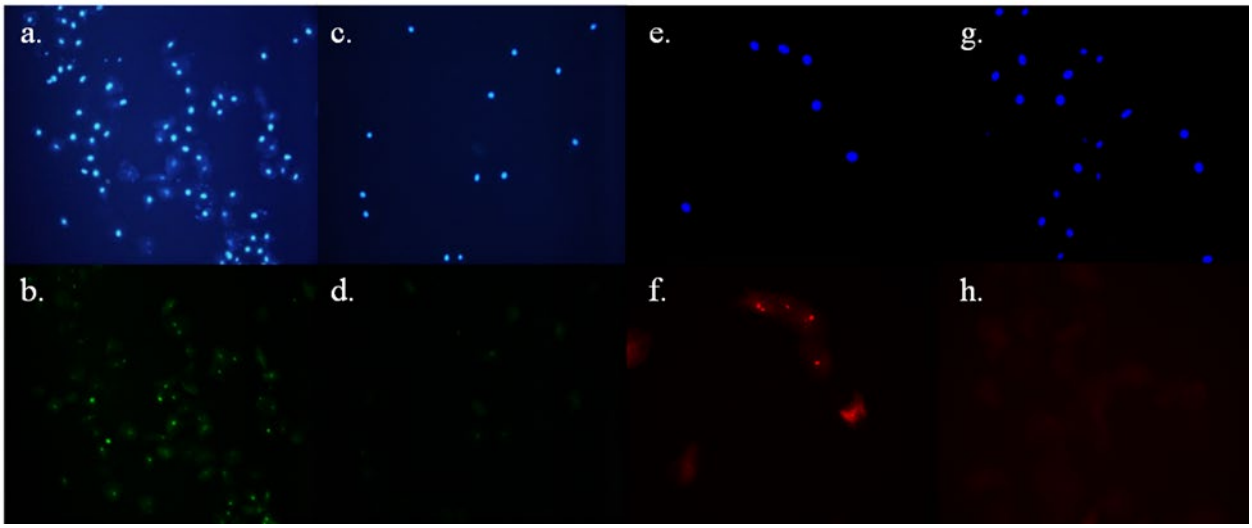

Figure 5: Male buccal epithelial cells stained with DAPI and green Y-probe visualized in the DAPI channel (a) and the FITC channel (b). Female epithelial buccal cells stained with DAPI and green Y-probe visualized in the DAPI channel (c) and the FITC channel (d). Male buccal epithelial cells stained with DAPI and orange Y-probe visualized in the DAPI channel (e) and the PE channel (f). Female buccal epithelial cells stained with DAPI and orange Y-probe visualized in the DAPI channel (g) and the PE channel (h).

ii. Staining Evaluation of Mixtures

After staining of mixtures of male and female cells, staining success was evaluated by adding a small amount of the sample to a slide and visualizing using a microscope. For the fresh 1:1 mixtures and 1:10 mixtures of male and female buccal epithelial cells, success was determined by visualization of any positive Y-probe staining. If Y-probe staining was present the sample proceeded to DEPArray analysis.

When assessing staining for 1:100 mixtures of fresh male and female buccal epithelial cells, counts were recorded due to the low expected staining efficiency of approximately 1% (Table 4). Staining efficiencies of 1.21% ± 3.25, 4.00% ± 11.21, and 3.33% ± 12.91 were observed for 3 1:100 runs. Similar results were obtained for the 1:1 and 1:10 mixtures were approximately 50% and 10% of the total cells had detectable Y-probe observed, respectively.

Table 4 Staining efficiencies of the in-solution CEPY Spectrum Orange method of 1:100 (male: female) fresh buccal epithelial cells.

Sample	# Positively stained cells	Total cell count	Fields of view	Expected staining efficiency (%)	Observed staining efficiency (%)
1:100 - 1	2	158	15	~1	1.21 ± 3.25
1:100 - 2	3	123	15	~1	4.00 ± 11.21.0
1:100 - 3	1	37	15	~1	3.33 ± 12.91

Aged and potentially low-quality samples may present a challenge for both the staining procedure and the subsequent DEPArray analysis. Four mixtures (1:10 male to female) were made using 10+ year-old buccal swabs were stained using the Spectrum Orange probe. Staining efficiencies were assessed by observing a small volume of the sample under the Zeiss Axioscope 5 fluorescent microscope, where there is an expectation that 10% of the cells will be male. Therefore, an observed staining efficiency of approximately 10% overall represents a near 100% staining efficiency for the male cells in the mixture. The staining efficiencies across the three mixtures were 13.00% ± 28.02%, 0.00% ± 0.00%, and 12.00% ± 31.55% were observed over 15, 10, and 10 fields of view, respectively (Table 5). The sample sizes were limited due to the use of the primary sample volume being applied to the DEPArray recovery process.

Table 5: The staining efficiency of the in-solution method with the Spectrum Orange Y-chromosome probe used with 1:10 mixtures (male to female) of aged buccal epithelial cell samples.

Sample	# Positively stained cells	Total cell count	Fields of view	Expected staining efficiency (%)	Observed staining efficiency (%)
1:10 - 1	4	55	15	~10	13.00 ± 28.02
1:10 - 2	0	26	10	~10	0.00 ± 0.00
1:10 - 3	2	31	10	~10	12.00 ± 31.55
1:10 - 4	0	45	10	~10	0.00 ± 0.00

iii. Cell identification, recovery, and profiling

Samples stained with Spectrum Orange Y-probe were loaded onto the DEPArray NxT or DEPArray PLUS. Figure 6 is an example of the staining profile on the DEPArray NxT, which depicts a male cell with strong Y-probe signal and a female cell (no Y-probe signal).

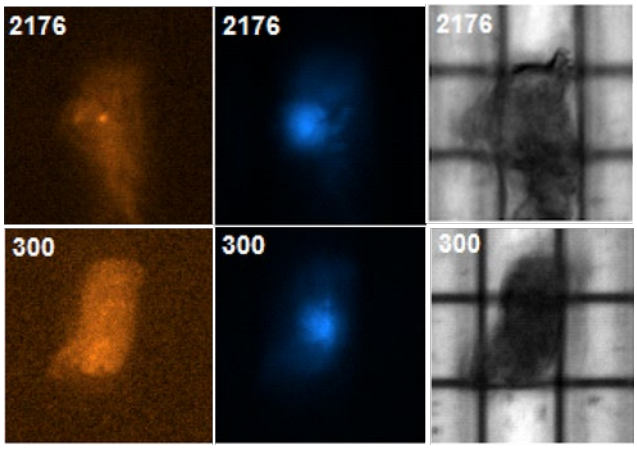

Figure 6: Top: Expected male buccal epithelial cell in the PE (left), DAPI (middle) and brightfield (right) channels on the DEPArray NxT. Bottom: Expected female buccal epithelial cell in the PE (left), DAPI (middle) and brightfield (right) channels on the DEPArray NxT.

The sensitivity of the DEPArray NxT system was a concern, where the optics and signal to noise were not as sensitive as a standard 400x fluorescent microscope. However, the DEPArray PLUS was found to improve signal intensity and thus increase sensitivity (Figures 7 and 8).

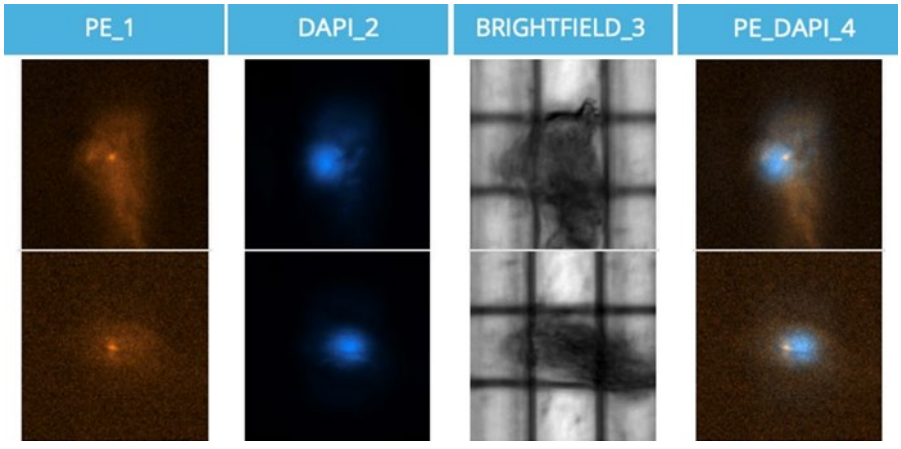

Figure 7: An example of two labeled male buccal epithelial cells (probe channel - PE_1, nucleus – DAPI_2, brightfield and PE/DAPI overlay) on the DEPArray NxT.

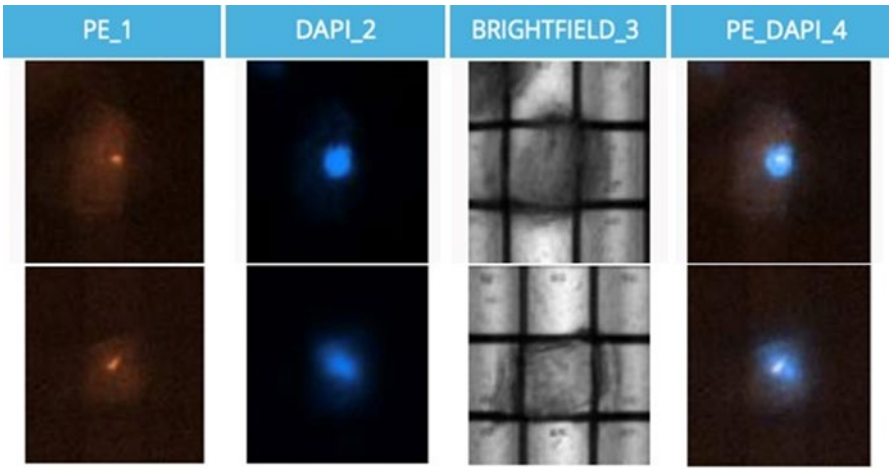

Figure 8: An example of two labeled male buccal epithelial cells (probe channel - PE_1, nucleus - DAPI_2, brightfield and PE/DAPI overlay) DEPArray PLUS.

The ability to identify and recover male (Y-probe stained) and female cells (unstained) on the DEPArray was first assessed through the recovery of single cells followed by profiling (seven runs in total, two 1:100 and five 1:10). Single cells were collected from five 1:10 mixture runs and two 1:100 mixture runs. 80% of expected male single cells generated a male profile (true positive) and 20% generated a female profile (false positive) (Figure 9 – top). 79% of expected female single cells generated a female profile (true negative) and 21% generated a male profile (false negative) (Figure 9 – bottom). The false positives may be a result of high background, a threshold is being investigated to avoid this. The false negative results were consistent with what was observed in the staining efficiency study.

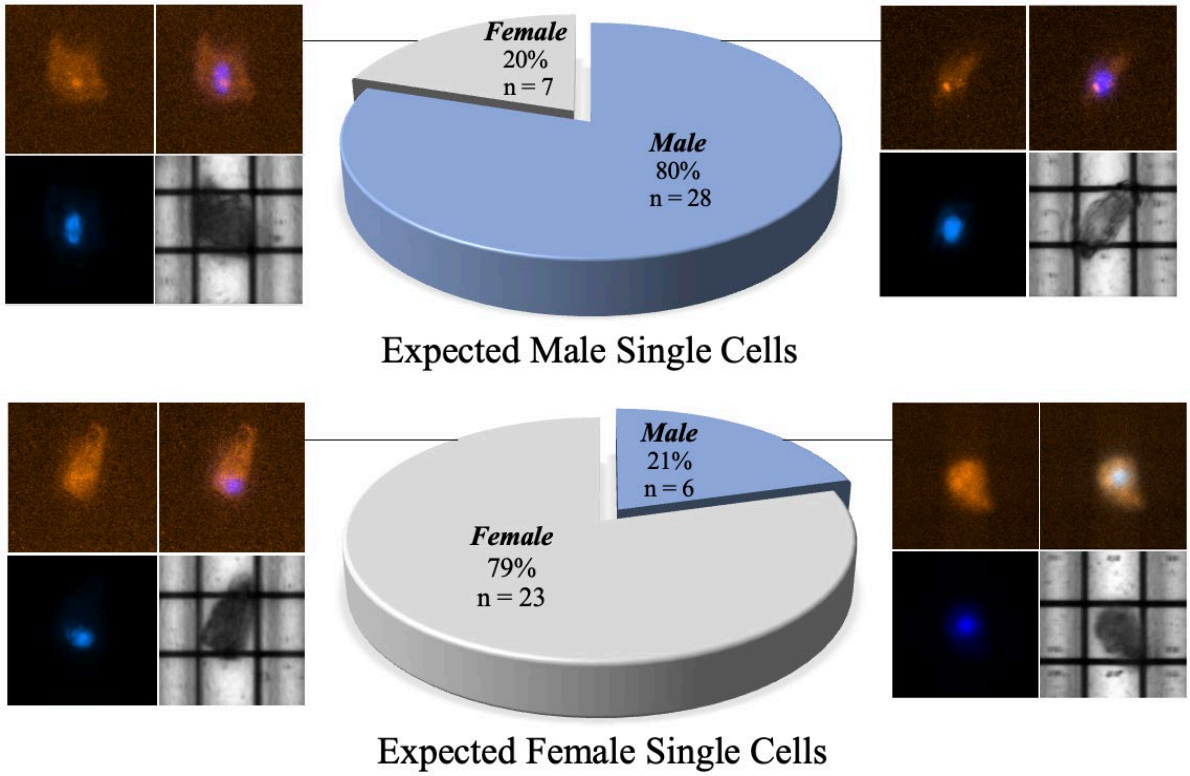

Figure 9: *Top* – A pie chart comparing the number of male to female profiles observed from expected single female buccal epithelial cells. *Top left* – an example of DEPArray images (PE, DAPI, PE and DAPI overlay, and brightfield) of one single buccal epithelial cell that produced a female profile; *top right* – an example of DEPArray images of one single buccal epithelial cell that produced a male profile. *Bottom* – a pie chart comparing the number of male to female profiles observed from expected female single cells; *bottom left* – DEPArray images (PE, DAPI, PE and DAPI overlay, and brightfield) of one of the single cells that produced a female profile, *bottom right* – DEPArray images of one of the single cells that produced a male profile.

Two mixture runs were carried out with male to female dilutions of 1:1 (Table 6). Genetic profiles of expected male cells recovered from the DEPArray were evaluated based on mean peak heights and the proportion of alleles present for the male and female donors. Single source male profiles were obtained from both 1:1 mixtures.

Table 6: Mixtures of 1:1 ratios of male to female cells, stained using the Y-probe staining method with expected male cells identified and recovered using the DEPArray NxT or NxT Plus and profiled using the PowerPlex Fusion 6c Human amplification kit (Promega).

Mixture (male : female)	Sample type	n (cells)	Routable cells	Male		Female		Donor or ratio of M:F
				Proportion alleles present	Mean peak height	Proportion alleles present	Mean peak height	
1:1 - 1	Expected male	5	739	41/46	146 ± 75	0/43	0 ± 0	Single source - male
1:1 - 2	Expected male	2	258	24/46	55 ± 35	0/43	0 ± 0	Single source - male

Next, five mixtures with 1:10 male to female dilutions were stained and separated using the DEPArray NxT (Table 7). Three of the 1:10 mixtures yielded single source male profiles. Two of the 1:10 mixtures resulted in mixed profiles with ratios of 1.1:1 and 0.85:1 male to female cells, respectively. The mixture ratios obtained were much more favorable for analysis than the original expected ratio of 1:10.

Table 7: Mixtures of 1:10 ratios of male to female cells, stained using the Y-probe staining method with expected male cells identified and recovered using the DEPArray NxT or NxT Plus and profiled using the PowerPlex Fusion 6c Human amplification kit (Promega). * 1:10 dilution of male to female fresh buccal epithelial cells extracted, amplified and detected without the use of the DEPArray (unseparated mixture).

Mixture (male : female)	Sample type	n (cells)	Routable cells	Male		Female		Donor or ratio of M:F
				Proportion alleles present	Mean peak height	Proportion alleles present	Mean peak height	
1:10 - 1	Expected male	3	507	35/46	108 ± 48	25/43	98 ± 36	1.1
	1:10 - 1*			40/46	307 ± 115	43/43	680 ± 177	0.45
1:10 - 2	Expected male	4	1273	46/46	555 ± 287	0/43	0 ± 0	Single source - male
1:10 - 3	Expected male	2	258	28/46	128 ± 68	0/43	0 ± 0	Single source - male
1:10 - 4	Expected male	6	450	27/44	82 ± 49	31/42	97 ± 51	0.85
1:10 - 5	Expected male	7	1165	44/44	220 ± 169	0/42	0 ± 0	Single source - male

Further, four mixtures with 1:100 male to female dilutions were stained and separated using the DEPArray NxT (Table 8). All four of the 1:100 mixtures yielded single source female profiles. One potential explanation for recovering false positives (female cells that appeared

labeled) is high level of background noise on the DEPArray NxT. This may be mitigated through improvements in the sensitivity of detection on the DEPArray or by testing alternative means to increase the intensity of the fluorescent signal of the probe, potentially through secondary immunostaining methods.. In addition, assessing the 1:100 mixtures on the DEPArray PLUS rather than the NxT could yield the improved results due to the better resolution on the PLUS. Additional data evaluating this remains that could not be included in this report but will be included in the final progress report. Figure 10 is comprised of examples of true and false positives and true negative cells detected and recovered using the DEPArray NxT from freshly collected and aged male and female cells.

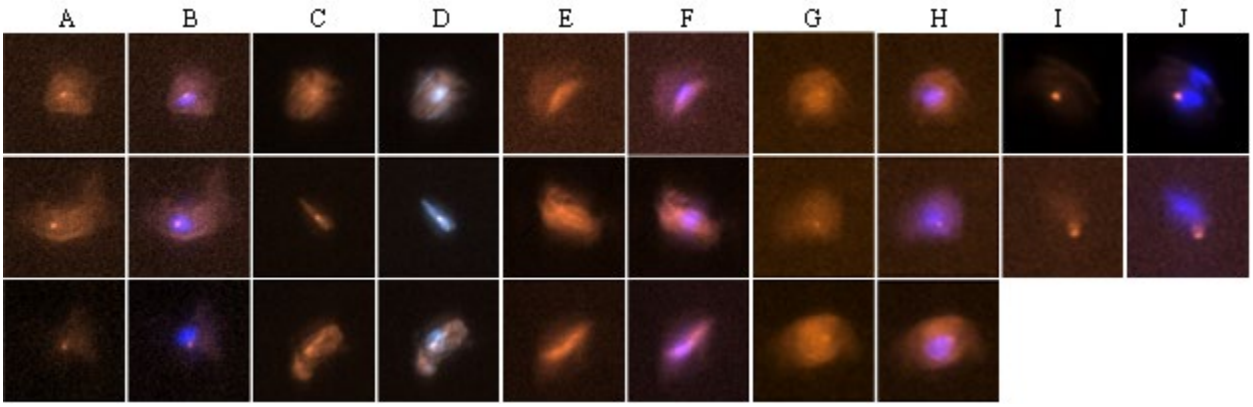

Figure 10: DEPArray detected and recovered cells : (A) freshly collected true positive – probe channel, (B) freshly collected true positive – probe and DAPI overlay, (C) aged true positive – probe channel, (D) aged true positive – probe and DAPI overlay, (E) freshly collected true negative – probe channel, (F) freshly collected true negative – probe and DAPI overlay, (G) freshly collected false positive – probe channel, (H) freshly collected false positive – probe and DAPI overlay (I) aged false positive – probe channel, (J) aged false positive – probe and DAPI overlay.

Table 8 Mixtures of 1:100 ratios of male to female cells, stained using the Y-probe staining method with expected male cells identified and recovered using the DEPArray NxT or NxT Plus and profiled using the PowerPlex Fusion 6c Human amplification kit (Promega).

Mixture (male : female)	Sample type	n (cells)	Routable cells	Male		Female		Donor or ratio of M:F
				Proportion alleles present	Mean peak height	Proportion alleles present	Mean peak height	
1:100 - 1	Expected male	1	648	0/44	0 ± 0	36/42	229 ± 131	Single source - female
1:100 - 2	Expected male	4	960	0/44	0 ± 0	42/42	223 ± 97	Single source - female
1:100- 3	Expected male	2	694	0/44	0 ± 0	37/42	109 ± 62	Single source - female
1:100 - 4	Expected male	7	1511	0/44	0 ± 0	33/42	123 ± 61	Single source - female

Three mixtures with aged 1:10 male to female dilutions were stained and separated using the DEPArray NxT. Two of the mixtures were successfully visualized and separated on the DEPArray PLUS but no profiles were generated. This was caused due to DEPArray reagent issues and was repeated with new DEPArray buffer (data included in the final progress report). The last 1:10 old mixture was separated with the DEPArray NxT. This sample yielded an expected male fraction (2 cells) and an expected female fraction (5 cells). The expected male fraction produced two failed injections, this sample will be injected again and re-assessed. The expected female fraction resulted in a mixed profile (data to be included in the final progress report).

iv. Conclusions

This study included optimization and evaluations of staining methods that could be used in a forensic setting and the use of the DEPArray as a means to identify and recover male cells from like-cell mixtures containing female cells. The method was demonstrated to have a staining efficiency of 80% or greater using freshly collected cells and translated well to the staining of

lower quality cells that will more closely mirror those observed in casework. In addition, the collection of male cells when recovered in groups has shown to be successful in 1:1 and 1:10 dilutions of male to female epithelial cells where female alleles were detected in two samples. Despite this, an improvement in the male to female ratio was observed and, therefore, still increased the ability to successfully deconvolute the mixture. The method, particularly with 1:100 dilutions appeared to be less robust however this is likely the result of the detection capabilities of the DEPArray NxT system. Improvements may be possible using the DEPArray PLUS and the improved optics on the system. This also can be mitigated using alternative means of recovering cells such as optical tweezers.

E. Applicability to Criminal Justice

The methods developed during this project will improve the sensitivity and subsequently the resolution of DNA analyses when analyzing samples composed of male and female like-cells. With the individual components of the mixtures separated, the resulting profiles are more easily interpreted (single source or a more easily interpreted mixture), require less time and fewer resources, and ultimately lead to increased confidence in the conclusions. For example, samples collected from sexual assault cases that involve vasectomized and azoospermic will have a significant quantity of female epithelial cells that overwhelms the small amount of male DNA present from male epithelial cells. Subsequent DNA analysis may produce little interpretable data regarding the male DNA profile, whether as a low-level contributor or an insufficient Y-STR profile. And, while crime labs have had success generating Y-STR profiles, these profiles are not searchable in the national DNA database. In cases with an unknown perpetrator the profiles do not generate any investigative leads. The study presents a method that can *target* and *recover* these male epithelial cells to obtain an interpretable male profile. This may result in

stronger statistical support for the analysts' results. It will also allow the profiles to be subsequently uploaded to CODIS to be searched against all national forensic and offender samples and, therefore, aid in generating investigative leads. The proposed method would also simplify the data interpretation, as manual or probabilistic mixture deconvolution methods may not be required. The benefits are not exclusively focused on sexual assault samples and extend to any sample with a male and female mixture of like-cells. For example, this method could be used in a case where a knife was collected from a female homicide victim's home. The majority of cells on the knife would be contributed by the female victim, however this method could separate male from female cells improving the chances of success. Other benefits include increased laboratory efficiency, where significant hours and resources that are applied to many rounds of DNA analyses that attempt to isolate the male profile can be avoided. Ultimately, the benefit extends to the greater criminal justice community through providing a new method that can potentially lead to higher quality results and conclusions which can positively impact the criminal investigation or trial.

F. Products

I. Scholarly Products

a. Publications – manuscript in progress
b. Technologies developed
    i. A protocol for preferential labeling of male epithelial cells in-solution like-cell mixtures

II. Dissemination Activities

a. Conference presentations

i. International Symposium on Human Identification (November 2022) Poster: *Sex-based targeted recovery of cells in a heterogeneous mixture: separating male and female like-cells.* Jonathan Hogg, Amber Vandepoele, Jeremy Dubois, Janine Schulte, Iris Schulz, Michael A. Marciano
ii. Louisiana Association of Forensic Scientists Spring Meeting (May 2022) Oral presentation: *Sex-based targeted recovery of cells in a heterogeneous mixture: separating male and female like-cells.* M. Marciano and J. Hogg.

iii. National Institute of Justice Grantees Meeting (February 2023 – upcoming)
Poster: *Sex-based Targeted Recovery of Cells in a Heterogeneous Mixture: Separating Male and Female Like-cells*. Jonathan Hogg, Amber Vandepoele, Jeremy Dubois, Janine Schulte, Iris Schulz, Michael A. Marciano

iv. Abstract Submitted – Green Mountain DNA Conference (July/August 2023). *Sex-based Targeted Recovery of Cells in a Heterogeneous Mixture: Separating Male and Female Like-cells*

b. *Media Coverage*

i. ISHI News. *Sex-Based Targeted Recovery of Cells in a Heterogeneous Mixture: Separating Male and Female Like-Cell*. M. Marciano and A. Vandepoele. https://www.ishinews.com/sex-based-targeted-recovery-of-cells-in-a-heterogeneous-mixture-separating-male-and-female-like-cell/

c. Continuing education

i. Preliminary results were presented to representatives of the Defense Forensic Science Center (USACIL, OCS and FDX) as a part of a continuing education talk.

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1.0 Purpose and Scope

1.1 This procedure will be a standard operating protocol for the use of the Abbott CEP Y Spectrum Orange DNA Probe to label male cells and recover the cells using the DEPArray NxT or PLUS

1.2 This procedure applies to the Bioforensics Laboratory located in Lyman Hall rooms 422 and 424.

2.0 Roles and Responsibilities

Role	Responsibility
Responsible Official	It is the duty of the Responsible Official (RO)/ Alternate Responsible Official (ARO) to ensure that this policy and procedure contain herein is read and understood by the appropriate laboratory personnel. The RO or ARO retains the ability to approve *Significant Protocol Deviations* when necessary. The RO/ARO must record the deviation in the ***Protocol Deviation log"*** It is the responsibility of the RO to ensure Bioforensics Laboratory Personnel complete the appropriate safety and bench training is obtained and completed in a manner that demonstrates competency. It is the responsibility of the RO and ARO to ensure the document herein is reviewed periodically to ensure continued relevance to the work being performed in the Bioforensics Laboratory.
Laboratory Personnel	It is the responsibility of the Bioforensics Laboratory Personnel to adhere to this policy and all other Bioforensics Laboratory and Syracuse University Policies. It is the responsibility of the Laboratory Personnel to obtain permission from the RO/ARO before implementing a *Significant Protocol Deviations*.

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3.0 Definitions/Acronyms

3.1 *FISH* – *f*luorescent in-situ hybridization

3.2 *RT* – room temperature

3.3 *DI* – deionized

4.0 Safety Considerations

4.1 None

5.0 Procedure-Test Method

5.1 Standards/Controls and Reagent Preparation

5.1.1 20X SSC Buffer Preparation

a. Combine:

i. 66 g 20X SSC

ii. 200 mL sterile DI water

b. Adjust the pH to 5.3 with HCl

c. Filter through 0.45 µm filtration unit

d. Store at RT (up to 6 months)

5.1.2 0.4X SSC Wash Solution

a. Combine:

i. 20 mL 20X SSC pH 5.3

ii. 950 mL purified water

b. Adjust the pH to 7.0 to 7.5 with 1N NaOH

c. Filter through 0.45 µm filtration unit

d. Store at RT (up to 6 months)

5.1.3 0.1% Triton x-100 (10% w/v) in 20X SSC

a. Combine:

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i. 1 mL 20X SSC pH 5.3

ii. 8.9 mL purified water

iii. 0.1 mL Triton x-100

b. Adjust the pH to 7.0 to 7.5 with 1N NaOH

c. Filter through 0.45 µm filtration unit

d. Store at RT (up to 6 months)

5.1.4 PBS with BSA

a. 1× phosphate buffered saline (PBS), 1% bovine serum albumin (BSA).

b. Weigh 0.05 g BSA and add 4 mL ice cold 1× PBS.

c. Mix until BSA has dissolved.

d. Make up to 5 mL with ice cold 1× PBS. Store at 4 ºC

5.1.5 1% BSA

a. Combine:

i. 0.05 g solid BSA

ii. 5 mL sterile dI water

5.2 Pre-Procedure Steps

5.2.1 DAPI dilution

a. Dilute the DAPI (1mg/mL) counterstain 1:10,000 if not yet diluted.

5.2.2 Set water bath to 73˚C.

5.2.3 Ensure the 1x PBS / 1% BSA solution used in step 5.5 is in the freezer at least one hour prior to the procedure.

5.3 Cell Suspension

5.3.1 Add 500µL of PBS into a lo-bind microcentrifuge tube and add the swab by cutting off the fabric portion into the tube.

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5.3.2 Place tube in thermomixer and incubate at room temperature at 300rpm for 20 minutes.

5.3.3 Spin at 600g for 3 minutes to pellet the cells, carefully remove the supernatant.

5.3.4 Take note of the size of the pellet.

5.4 Fixation

5.4.1 Make 2% (formaldehyde) using 1% PBS.

5.4.2 Add 100µL of fixative to each tube slowly, pipet up and down for 15 seconds, and then add an additional 100µL and pipet up and down for 15 seconds again.

5.4.3 Incubate for 10 minutes at room temperature.

5.5 Cell Permeabilization

5.5.1 Add 500 µL ice cold 1x PBS / 1% BSA.

5.5.2 Spin at 600g for 3 minutes and remove and discard supernatant.

5.5.3 Resuspend cells in 500 µL ice cold 1x PBS / 1% BSA.

5.5.4 Spin at 600g for 3 minutes and remove and discard supernatant.

5.5.5 Resuspend cells in 200 µL 0.1% Triton x-100.

5.5.6 Spin at 600g for 3 minutes.

5.5.7 Remove and discard supernatant.

5.6 Hybridization of DNA to probe

5.6.1 For a 0.35x concentration (final volume = 40 µL):

a. Add 38.65 µL hybridization buffer to each tube.

b. Transfer the sample(s) into a 0.2mL PCR tube(s).

c. Add 1.35 µL CEP Y probe to each tube.

NOTE: all tubes that have the CEP Y probe should be kept out of light for the remainder of the protocol

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d. Place samples in a 73˚C water bath for 5 minutes

e. Rotate samples on a laboratory tube rotator (rotisserie) at room temperature overnight.

   i. **IMPORTANT**: Ensure that the tubes are fully covered in foil and/or kept out of direct or indirect light.

f. Warm 0.4x Wash SSC Buffer at 73˚C for 10 minutes.

g. Transfer samples into lo-bind microcentrifuge tubes.

h. Wash the PCR tubes with 200 µL warmed 0.4x Wash SSC Buffer and add to the samples.

i. Incubate cells at 73˚C for 2 minutes to facilitate removal of excess probe that may have specifically bound to sequences partially homologous to target sequence.

j. Add 200 µL ice cold 1% BSA to cells to quickly drop the temperature and prevent clumping of cells.

k. Centrifuge at 600g for 3 minutes.

l. Remove and discard supernatant.

5.7 DAPI counterstain and slide application

5.7.1 Resuspend the cells in 100 µL ice cold 1x PBS / 1% BSA.

5.7.2 Spin at 600g for 3 minutes.

5.7.3 Remove and discard supernatant.

5.7.4 DAPI staining:

a. Add 25 µL of a 1:10,000 dilution of DAPI and incubate for 40 minutes at RT

b. Centrifuge at 600g for 3 minutes.

c. Remove and discard supernatant.

5.7.5 Post - DAPI counterstain

a. Add 100 µL 1% cold BSA to each sample.

b. Centrifuge at 600g for 3 minutes.

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c. Remove and discard supernatant.

d. Add ~ 30 µL if ice cold 1% BSA to each sample.

e. (**Optional**) Apply ~ 5 µL of the sample to microscope slide and let air dry in the dark. The microscope slide should be used to check staining efficiency. The rest of the sample should be kept in suspension.

f. Add 3 µL Vectashield mounting media to microscope slide and coverslip.

NOTE: The sample can either be stored covered in foil and out of light at -20° C or it can be run on the DEPArray.

NOTE: If the sample will be run on the DEPArray the following day ensure that the room humidity is at least 50%. If it is not run humidifiers the day prior.

5.8 Stain Quality Check with Fluorescent Microscope (optional)

This procedure is to check the quality of the staining. It is critical that the DAPI staining is visible and stained a majority of the cells present. The number of cells stained with the Y probe will vary because it is dependent on the number of male cells in the sample. The internal protocol for the fluorescent microscope available at the laboratory should be used. See table 1 for recommended filters.

Table 1: Probe emission and excitation spectra with recommended filters.

Fluorescent Probe	Excitation Peak	Recommended Channels	Excitation wavelength (nm)	Emission wavelength (nm)
Spectrum Green™	497	FITC	469	510
Spectrum Orange™	559	PE/Cy3	546	581
DAPI counterstain	367	DAPI	376	447

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5.9 DEPArray Protocol

Prior to preparing the sample for loading onto the DEPArray turn the sign into the DEPArray instrument account (on the DEPArray onboard computer) and select start 'Start. Instrument tests will be automatically performed. If they do not pass access the Menarini portal and complete a ticket. Note, low humidity (under 50%) is a common error experienced during the winter months.

5.9.1 Sample Preparation - *DEPArray Buffer Washes*

The DEPArray buffer washes are to remove the buffers/reagents that were used to stain the sample and to impart a negative charge on the cells to permit recovery on the DEPArray instrument).

a. Remove the DEPArray Buffer from freezer and thaw to room temperature
b. Place the DEPArray Buffer into a liquid bath sonicator and degas for 10 minutes (600s) - (see '*TRA_TUX_130-R1-DEPArray buffer for fixed cells degas procedure for use'* for further details).
c. Add 500 µL of DEPArray Buffer to the sample and centrifuge in a centrifuge. with a rotating bucket rotor for 3 minutes at 600g.
d. Once complete, remove supernatant leaving ~50 µL of buffer.
e. Repeat steps c. and d. 2 additional times for a total of 3 washes.
f. (**Optional**) Count the cells using a hemacytometer.
    i. The maximum number of cells loaded in the DEPArray forensic mode is 6000, with an optimal number between 2,000 to 3,000.

5.9.2 Sample Preparation - *Cartridge Loading*

The DEPArray Buffer and sample should be added to the cartridge using only Menarini approved micropipettes and tips to ensure proper fit into the cartridge ports. Follow the DEPArray Cartridge loading procedure (summarized below).

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a. Open a new cartridge, making sure to save the outer packaging.
b. Load 2.5 mL of the DEPArray Buffer into port “B” slowly, make sure no air bubbles are introduced to the cartridge lines.
c. Load 12 µL of the sample into port “S” slowly, making sure no air bubbles are introduced into the cartridge lines.

5.9.3 DEPArray System Setup and Cartridge Loading

a. If the instrument is not powered on, turn the system on and sign into user account and select ‘Start’.
b. Instrument tests will be automatically performed. If they do not pass access the Menarini portal and complete a ticket.
c. The instrument door will then open, and the cartridge can be placed into the drawer.
d. Wait for the prompt and scan the barcode of the gray cartridge packaging.
e. Skip the buffer labeling step however name the run (e.g., date_initials).
f. Select mode ‘Forensics RUO’ set to ‘fixed’ and ‘enable’
g. Right click on ‘Chip Scan’ and add PE, DAPI and Brightfield to be the selected channels.
h. Right click on PE and select ‘take picture PE’ and set PE to faint signal
i. Right click on DAPI and select ‘take picture DAPI’ and ensure DAPI set to bright signal.
j. Right click on Brightfield and select ‘take picture Brightfield’.
k. Add a second PE & DAPI channel (PE1 and DAPI1) and select ‘take picture’ for each.
l. Begin Sample Load
    i. Wait until Sample load reaches 100% to proceed to the next step. Note, it typically pauses at 7% and ~70%.

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5.9.4 Sample Scan

a. Monitor the sample scan until several fields with cells are scanned.

b. Pause the sample scan and select cages in the bottom right of the main chamber (most cells are located around the outer corners of the chamber).

   i. Set parameters (exposure, camera gain, lamp intensity, offset) to best provide a visible signal in both PE (PE1) and DAPI (DAPI1) channels.

   ii. Set an offset at a different µm for the PE1 and DAPI1 channels so that there are 2 focal points for the cells (e.g., PE and DAPI can be set at 55 µm and PE1 and DAPI1 can be set at 35 µm).

c. Resume Sample Scan until complete (~30-40 minutes).

5.9.5 Cell Selection

a. Select ‘parameters’ on the top left and select signal and max intensities for PE and DAPI channels to set up a table with all cells on the left side of the screen.

b. Select the channels on the bottom of the screen (APC, PE, PE1, DAPI, DAPI1, Brightfield, an overlay of PE and DAPI and an overlay of PE1 and DAPI1),

   i. Set the colors to your preference (PE = orange, DAPI = blue).

c. To create new tables, select ‘New Table’ at the top of the panel.

   i. For example: Table 0 – for ‘male only cells’ – single cells with signal in PE channel; Table 1 – for ‘mixed clumps’ – multiple cells with at least one signal in the PE channel; Table 2 – ‘unknown’ cells that could have signal in PE channel, but not distinct; Table 3 – ‘female only’ – single cells with no signal in PE channel.

d. Sort the Cell Panel by PE intensity (descending) by selecting the PE column header.

e. Manually review the cells and use a Hot Key to send the selected cell to its intended table.

   i. The Hot Key is the number next to its associated table in brackets (e.g.,

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Table [0] has a Hot Key of 0. Group the cell with its intended table.

ii. To deselect a cell from the table, press the associated Hot Key again.

f. (**Optional**) **Cell Panel – Saving images**

i. Once the cells are in their respective groups, navigate to the Cell Panel and Select a table to view the cells in the selected table.

ii. To save, select the camera icon on the left, choose the .tiff extension, choose each channel individually, then select the icon on the bottom left to save it to the run file, then select OK.

iii. Repeat for all channels that are to be saved (PE, DAPI, PE1, DAPI1, Brightfield, overlay of PE/DAPI and overlay of PE1 / DAPI1).

5.9.6 Routing

Note : 'Group 1' is generically used as a label for the groups of cells recovered in the each of the tables set in previous steps.

a. Select the '+' button next to the table to view the selected cells with their Cell ID.

b. On the bottom left, open the shutter and look at the live view of the chamber.

c. Set the parking speed to 1000ms (this slows down the speed of the routing but minimizes the chance the cells are lost during the routing process).

d. Select on obstacles to see any possible obstacles that surround the cells.

i. Select on Group 1 and select move to parking and press the start button to begin the parking process.

ii. Observe the cells in the live configuration and pause the routing if any cell is lost from its Cell ID during this stage.

e. Repeat for all tables

i. Park each group individually.

ii. To do this, individual cells can be selected and then moved to parking.

iii. Take a picture of the live configuration once the cells are parked to see if there was any cell loss.

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5.9.7 Recovery

a. On the top left, set up recovery configuration and select 200 µL recovery tubes.

b. Navigate to the option to open the recovery support (On the bottom left of the pop-up icon).

   i. To do this, select the Trays on the DEPArray screen, then open recovery

   ii. Load the proper amount of 200 µL PCR recovery tubes with an extra in A1 for the primer (the primer is to start to reagent drop to ensure proper elution of the cells). Tubes should be placed in every other column and every other row.

   iii. For example: 5 tubes total → A1 – primer; A3 – Group 1; A5 – Group 2; A7 – Group 3; A9 – Group 4.

c. The number of drops is automatic but ensure the proper number of drops under the recovery schematic are selected (see DEPArray User Guide for more details).

d. To begin recovery, right click on A1 and set the primer.

   i. This will wash the main chamber of the remaining cells and set the primer in A1.

e. To recover the Groups, select Group 1 and drag it to A3.

f. Repeat for the other Groups.

   i. Note, if the group is split and identified with different colored cells, they can be dragged individually based on the color to separate recovery tubes.

g. Once the recovery support complete, right click on Group 1 in the center of the screen and move to recovery.

   i. Repeat for the rest of the groups.

h. Once recovered, remove the support tubes.

i. Stop the run and the cartridge can be discarded and system can be shut down.

j. Proceed to volume reduction (See Menarini Volume Reduction Manual).

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6.0 Referenced Documents

Policies, Procedures, and Work Instructions	Templates & Forms	Examples
DEPArray NxT User Manual	Version 3.0	
W_MKT_051 DEPArray™NxT Volume Reduction Protocol for Fixed Cells Instructions for Users	Version 1.1	
TRA_TUX_130-R1-DEPArray buffer for fixed cells degas procedure_for use	Rev. 1	

7.0 Records

Record	Responsibility	Location	Retention	Disposition
NA	NA	NA	NA	NA

8.0 Standards Mapping

Standard	Requirements
NA	**NA**

9.0 Revision History

Date	Item	Description
NA	NA	NA

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