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Illuminating touch deposits through cellular characterization of hand rinses and body fluids with nucleic acid fluorescence

Abstract

Forensic DNA typing from touched or handled items in routine casework is increasing as the sensitivity of detection techniques improves. Our understanding of the cellular/acellular content of touch deposits and the origins of the DNA therein is still limited. This work explores the cellular content of rinses from washed and unwashed hands, as well as saliva, nasal and eye washes which could be sources of transferred DNA onto hands. Flow cytometry and microscopic examination were used to detect granularity, size and nucleic acid fluorescence data. Cellular content did not vary significantly within an individual, although some differences were observed between donors. Saliva contained populations of nucleated epithelia as well as smaller cells and debris, all positive for DNA. Hand rinses consisted almost entirely of anucleate corneocytes, many of which also stained positive for nucleic acids. These data raise questions about shed corneocyte DNA content previously assumed to be negligible.

Keywords: touch DNA; DNA localization; flow cytometry; body fluids; cellular content; skin cells

1. Introduction

"Touch DNA" refers to genetic material left behind after handling an object or a person. It is now regularly used as a part of forensic DNA casework. As methods of recovery, processing and detection improve, so does the ability to generate DNA profiles from smaller residues of deposited DNA [1]. Despite its frequent role as legal evidence, touch DNA itself is poorly understood. In particular, there has been limited direct investigation into the cellular composition of touch deposits [2, 3].

Touch DNA is often described as coming from shed skin cells [4, 5], yet there are several possible sources to consider. Firstly, the outermost layers of skin consist of terminally differentiated keratinocyte cells called corneocytes, which lack nuclei and organelles, though they may retain DNA [6, 5]. Some studies have shown cell-free, or extracellular, DNA to be a viable alternative source of touch DNA [7, 8, 9]. Fragmented cells or free nuclei from degraded cells have been proposed as another source [10, 11, 12]. Finally, it is also possible that the DNA deposited by handling does not originate in the hands at all but may accumulate after contact with richer sources of nucleated cells such as nose, eyes or mouth [13, 14].

The localization of DNA to one or more of these sources could have implications for how touch DNA is collected, analysed, and interpreted in forensic contexts. For example, if nucleated cells secreted through sweat pores are found to be the primary DNA source, then current extraction methods can be considered appropriate. Improvements could simply entail optimization for maximum recovery efficiency. However, if cell fragments or free nuclei are contributing to recoverable touch DNA deposits, size-based filtration could represent a suitable extraction alternative. If touch deposits consist of nucleated cells accumulated from elsewhere onto the hands and later deposited, then our understanding of DNA transfer potential may need to change to acknowledge that all touch DNA is in essence transferred DNA. If anucleate corneocytes or cell-free DNA are significant sources of touch DNA, then further research into the DNA present therein may be required. Current STR amplification methods may not be appropriate for the potentially fragmented DNA in corneocytes or

cell-free fractions, thus -specialized lysis, purification or amplification methods may need to be developed. A more complete understanding of the composition of touch DNA deposits will improve the ability to reliably use this type of forensic evidence in casework.

Existing research has begun to explore the relative contributions of cell-free and cellular components of touch deposits [8, 15]. Methods in use are mostly centrifugal separation and occasionally use of filter purification [9, 7]. Other separation techniques, such as laser-capture microdissection, have also been used in forensic analysis. These are most useful in separating visually distinct cell types or particulates, such as sperm cells, epithelia, white blood cells, hair follicles and debris [16]. When separation technology is applied to forensic samples it usually serves to separate individual contributors' cells to aid in downstream DNA mixture deconvolution, making DNA profiles clearer to interpret [17]. This has been reported with immunomagnetic beads [18, 19], microfluidic chips [20], and dielectrophoretic arrays [21]. However, the resolution of constituents within a single-source sample is less queried. Some success has been shown in using a microscope to guide collection of individual "cellular microparticles" collected on tape lifts for DNA analysis, which could be useful when exploring touch deposit components, although inquiry was not made into relative DNA contributions by cell type [3]. Successfully separating the components of touch deposits and identifying the origin of the DNA therein will resolve a fundamental uncertainty about the nature of these samples which are used in criminal casework every day.

This present investigation used flow cytometry for the separation of touch deposit components and the localization of DNA. Although widely used in biomedical fields, limited studies have utilized this technique in forensic research. Focus has been primarily on sexual assault or blood mixtures, attempting to resolve constituents by cell type (sperm or epithelia or leukocytes) or by human leukocyte antigen (HLA) probes [22, 23, 24]. The goal has been to simplify downstream DNA mixture analysis by separating contributors early by distinct fluid source. Existing flow cytometric work on touch samples has also focused on DNA mixture deconvolution by parsing individual autofluorescent optical signatures using antibody probes [25]. The work presented here used flow cytometry of known single-source samples to resolve constituents of controlled touch deposits. Nucleic acid-specific dyes were used to localize DNA within different cell-populations. This work begins to define the cellular contents of a touch deposit and determine where within them DNA originates.

This investigation contained three analytical phases: first, characterization (based on size and granularity data from flow cytometry) of a single individual's hand rinses and body that might be contributing to touch deposits via transfer from eyes, nose and mouth. The second phase examined whether these cellular content patterns varied significantly either within an individual on various days or between individuals. Finally, a series of fluorescent nucleic acid dyes was used to determine whether DNA was located in a particular cellular or subcellular population within hand rinses and the potentially contributing body fluids. This phase was paired with microscopic examination and intended to address the origin of touch deposits' cellular DNA.

2. Materials and Methods

2.1 Sample Collection

Donors were volunteers from the King's College London (KCL) community, and sample collection approved by KCL Biomedical Sciences, Dentistry, Medicine and Natural & Mathematical Sciences (BDM) Research Ethics Subcommittee (HR-17/18-5500).

Unwashed hands. Six donors rubbed their unwashed hands lightly together as 6 ml of sterile, filtered Phosphate Buffered Saline (PBS) was applied to them 2 ml at a time. The rinse was collected in a sterile weighing boat and transferred to 1.5 ml microcentrifuge tubes.

Washed hands. Following the above collection, participants washed their hands with soap and thoroughly rinsed them in tap water for 1-2 min and then allowed them to air dry without any contact. The 6 ml rinse collection was repeated and samples transferred into 1.5 ml microcentrifuge tubes.

Saliva. The same donors, who had not consumed food or drink for at least 1 hr prior to collection, were asked to rinse their mouth briefly with water. Donors were asked not to spit but to allow saliva to accumulate in their mouth for several minutes before deposition. This was done to minimize collection of scraped buccal cells and focus collection on the shed populations most likely present in transferable saliva. Subsequently 3 ml of saliva was then deposited over several minutes by the donors into sterile weighing boats and diluted with 3 ml of PBS. This reduced the viscosity and facilitated further processing.

Nasal lavage. A total of 6 ml of PBS was applied in 2 ml increments using sterile disposable pipettes by volunteers to their own nasal cavity (both nostrils) to create nasal lavage under researcher supervision. Run-off was collected in sterile weighing boats as it exited the nasal cavity prior to contact with external skin.

Eye wash. Sterilized eye rinse cups (Boots) were used by volunteers to flush 6 ml of PBS in 2 ml increments onto their opened right eye and collect the run-off. All run-off PBS, containing the cellular samples, was then transferred to 1.5 ml microcentrifuge tubes.

Cell culture. Immortalised normal human keratinocytes were cultured in high glucose Dulbecco's Modified Eagle Medium (Sigma) supplemented with 10% v/v Fetal Bovine Serum (ThermoFisher), 30% v/v Ham's F12 (Sigma), 1% v/v L-Glutamine (Sigma), 50 U/ml penicillin and 50 mg/ml streptomycin (Sigma), 1% v/v Rheinwald Media with growth supplement and incubated at 37 °C with 5% humidified CO_2. These adherent cell cultures were detached with 0.05% Trypsin-EDTA (Invitrogen) for 20 min at room temperature and used as a positive control for cell staining.

Each of the 5 body fluid samples was collected once from 6 donors (n = 30). Each sample was divided into 5 subsamples for processing with different conditions. For three of the donors, intraindividual variability was also evaluated by repeating body fluid collection on 4 additional days (5 different days in total, n = 75).

2.2 Nucleic Acid Staining

Aliquots (350 µl) of each sample type (unwashed hands, washed hands, saliva, nasal lavage, eye wash, control keratinocytes) were mixed with each of the following staining conditions: thiazole orange (TO; 84 nmol/L), propidium iodide (PI; 2.15 µmol/L), DiamondDye (DD; 1X), combined TO/PI (84 nmol/L TO, 2.15 µmol/L PI), and PBS only as unstained negative control. TO concentration is as indicated in manufacturer's guidelines (BD Cell Viability Kit, BD Biosciences) for mammalian cells. PI concentration is 0.5X of manufacturer's suggested concentration (BD Biosciences) due to observed over-saturation in early trials (data not shown). DD sold by manufacturer (Promega) at 10,000X stock in DMSO and was diluted to 100X in PBS; 1X concentration used based on early trials showing oversaturation at higher levels (data not shown).

Each condition (sample type and nucleic acid stain combination) was set up in triplicate in separate wells of a U-bottomed 96-well plate and incubated in the dark at room temperature for 5 minutes. Subsequently, three aliquots of 3 µl were removed from each sample for microscopic examination and the remaining sample volume was retained in the 96-well plate wells for flow cytometry. A plate

well containing only 350 µl PBS and a plate well with 350 µl of each staining condition in PBS alone were also included as negative controls for contamination events and background fluorescence.

TO is a cell permeant cyanine dye which functions as an intercalating fluorophore, while PI is also an intercalating dye but will not permeate live/intact cells. Both will fluoresce upon nucleic acid binding and are widely used in microscopy and flow cytometry. Used together, they are considered a cell viability assay allowing a colour distinction between live and dead cells, or intact and compromised cells. DD is an external groove DNA-binding dye intended for visualization of nucleic acids in gels. Its use in quantitative PCR and microscopy for touch DNA has been reported in the forensic literature [26, 5].

2.3 Flow Cytometry

Each sample was run on a CytoFlex flow cytometer (Beckman Coulter) with a 96-well plate loader fitted with 488 nm and 638 nm lasers; voltages for all tests were set at 50 mW and 80 mW, respectively. All samples were run with a flow rate of 10 µl/min. Forward Scatter (FSC) and Side Scatter (SSC) data were collected for 10,000 events. Gating parameters were not implemented as debris-sized events were potentially of interest and therefore not removed. Data analysis was conducted with CytExpert 2.0 software provided by Beckman Coulter. All measurements were made using the 488 nm laser.

A size ladder (pooled 1 µm, 2 µm, 4 µm, 6 µm, 10 µm, and 15 µm beads) was run with one of each sample type and with PBS alone in order to accurately size the cellular and subcellular events in each run and allow for absolute measurements.

Forward Scatter (FSC) v Side Scatter (SSC) plots show size measurement against granularity of each particulate in the sample. For cellular content characterizations, each plot was divided into four size categories expected to reflect biological constituents: < 6 µm (subcellular fragments and debris), 6-10 µm (subcellular fragments, including potential free nuclei), 10-15 µm (subcellular fragments and small cells, e. g. leukocytes), >15 µm (intact cells including epithelial cells and terminal keratinocytes (corneocytes)). For DNA localization, each sample's detectable autofluorescence was measured on an unstained well and then a threshold was set on the stained sample to include all particulates above this level of fluorescence.

2.4 Microscopic Examination

Three 3 µl aliquots of each sample was spotted onto a poly-L-lysine coated slide. Each condition was spotted in triplicate and allowed to air dry in a dark environment. These were visualized with Zeiss fluorescence microscope using FluoroSave (CalBioChem) as mounting medium. The images were captured at 200x magnification with AxioCam 4.8 software.

3. Results and Discussion

3.1 Flow Cytometry

3.1.1 Cellular Content Characterization by Body Fluid

It was initially hypothesized that the cellular make-up of each body fluid might be sufficiently distinct as to create recognizable cell populations on these plots. If that was the case, the intent was to determine whether these recognizable populations could be detected in unwashed hand samples. This would demonstrate that unwashed hands could be accumulating and redepositing detectable amounts of transferred cells from other body fluids. This would be further supported if the washed hands did not display the same cellular populations. Since the focus of this inquiry is cellular contents of touch deposits, the section representing keratinocytes (above 15 µm) is of particular relevance.

All sample types display a high density of small, low granularity events presumably representative of debris or very small fragments, followed by a more spread out population of larger, more granular events reflective of the intact cells (Figure 1). These results comport with previously published reports of cell-size corneocyte populations in touch deposits (15-50 µm) as a distinct cluster from small-sized events (generally below 10 µm) [27], which could include debris, cell fragments, free nuclei or even intact leukocytes present in body fluids which could have transferred [28, 29, 30].

The FSC v. SSC plots in Figure 1 for saliva and both washed and unwashed hand rinses contain a relatively large population of cells above 15 µm in diameter spatially distinct from the small-sized debris. Eye wash and nasal lavage samples had consistently low numbers of large cellular events as well as being less cell-dense overall (mean time to accumulate 10,000 events was 5:56 min for nasal lavage and 9:17 min for eye wash with one sample reaching the 10:00 min time maximum before 10,000 event were measured; mean time for saliva, washed hands and unwashed hands were 2:50 min, 0:58 min, and 0:50, respectively).

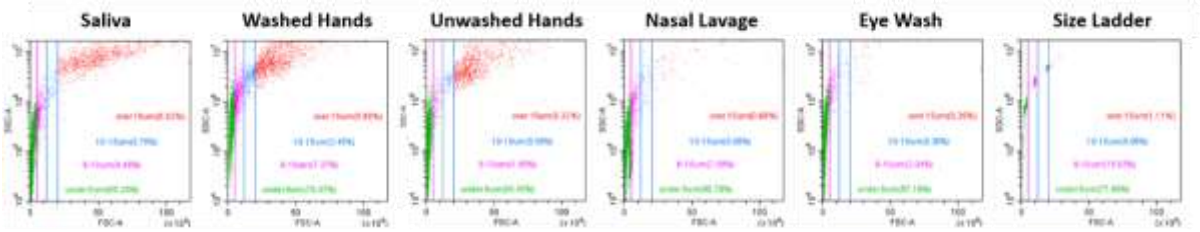

Figure 1. An exemplar FSC v. SSC plot for each sample type from a single individual run on a flow cytometer. The X axis represents Forward Scatter (size) and the Y axis represents Side Scatter (granularity). Samples shown divided into coloured size sections (green: events under 6 µm, pink: 6-10 µm, blue 10-15 µm, red: over 15 µm).

The mean proportion of events by size section for three replicates of a single donor on a single day was calculated and is shown in Figure 2. The proportions of events were compared between each sample type with a paired t-test. The proportion of large cell events (over 15 µm) is not significantly different between saliva and either hand rinse or between the washed and unwashed rinses ($0.11 \leq p \leq 0.88$), suggesting a considerable population of epithelia in all three sample types. However, the large cell proportion is significantly larger in each of these sample types than in either nasal lavage or eye wash ($p \leq 0.03$), which do not differ from each other ($p = 0.07$). This may indicate that epithelial cells are more readily shed from mouths and hands than from nasal cavity or eyes, or it may simply reflect differences in the amount of surface contact or friction used during collection.

Debris is consistently the highest proportion (over 60%) of every sample, which is expected due to the lack of gating. Often flow cytometric analysis would gate out, or exclude, this particulate debris as irrelevant to the analysis of cultured cell populations. All particulate sizes were included in this

analysis because hand rinses remain uncharacterised and the DNA content of small cells or cell fragments was of interest.

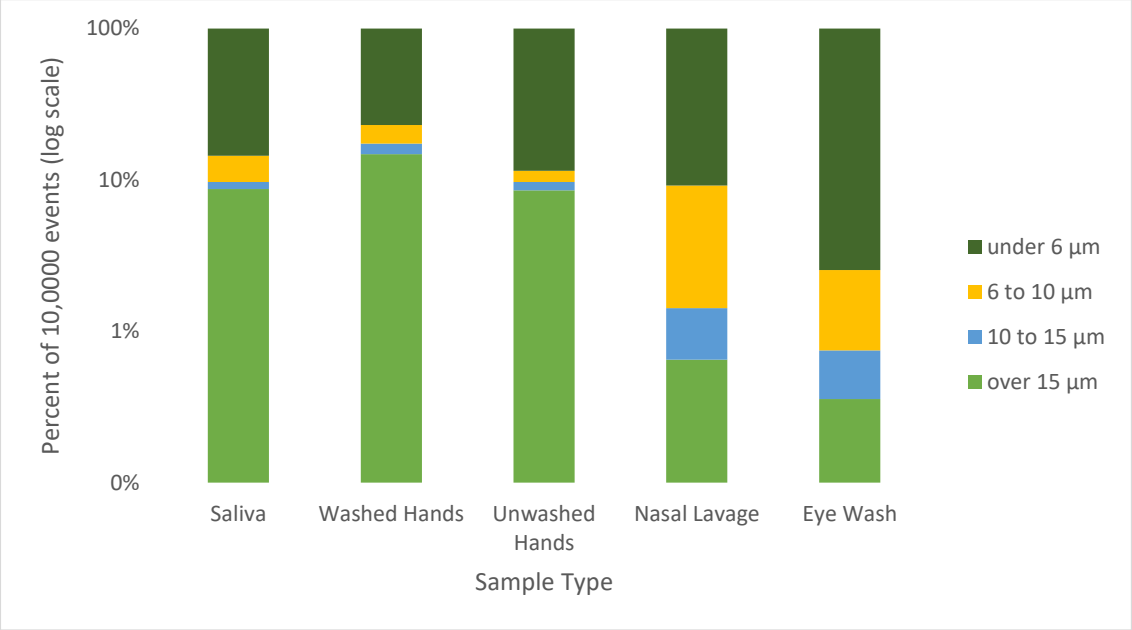

Figure 2. The mean proportion of events by size section for 3 replicate wells for a single donor. The proportion of large cell events is not significantly different between saliva or either of the hand rinses, although both nasal lavage and eye wash have significantly smaller cellular populations.

The smaller number of cell-sized events in nasal lavage and eye wash samples may result from the lack of friction used to collect these samples compared to hand rinses. However, less friction was used for saliva collection, so the cell density may suggest that mouth mucosa sheds cells more readily than that in the nose or eyes. It was thought that using a wash in the mouth (rather than simply collecting fluid as it accumulated) might introduce more friction into collection and thus cause additional buccal cell shedding. This is why it was avoided for saliva collection. It was not practical to collect sufficient volumes of any of the other sample types without a wash. The low cell density in nasal lavage and eye wash suggests that washing may not introduce excessive friction.

The cell distributions of these five sample types were not considered distinctive enough to allow a pattern to be detected in a mixed sample. For example, no cell cluster in nasal fluid or saliva was distinctive enough by size/granularity alone to make it recognizable if unwashed hands contained traces of those fluids. Thus, cell distribution alone was not able to shed light on the potential for unwashed hands' touch deposits to consist of transferred cellular content as was originally hypothesized.

To assess the impact of day to day variation on an individual's shedding of cells within each of the examined body fluids, three donors were sampled on five separate days and the FSC v. SSC plots were compared (see Figure 3). The detected cellular and fragmentary events were sectioned into size intervals and proportion of events in each section was compared across 5 days with a single factor analysis of variance. When a single sample type, washed hands for example, was examined across multiple collection days, no significant difference in mean percentage of large cellular events was observed ($p > 0.05$). This was true for all sample types.

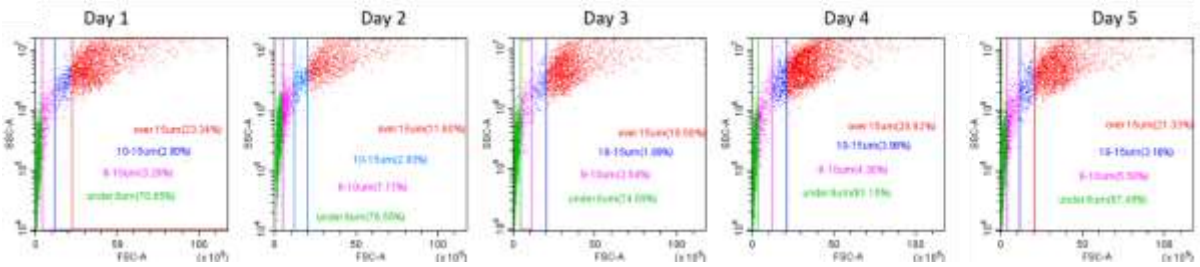

Figure 3. A single donor's washed hands rinse collected on 5 separate days. The mean event percentage in cellular size ranges was calculated from triplicate analyses and compared across five collection days.

Consistent results were observed between different collections of the same body fluid. This consistency was also observed between replicate runs of each sample. These results suggest very limited variability within a single individual.

3.1.2 Cellular Content Characterization by Donor

Relative proportion of cells within a sample type, as shown in event distributions on FSC v SSC plots, varied somewhat between different individuals. An exemplar replicate of washed hands from each of 6 donors are shown in Figure 4. The same size ranges are delineated. Percentage of large cell events above 15 µm ranges from 7.4%-57.2% among individuals. The mean of the maximum differences between any two donors' large cell population percentages is 30.9%. This is higher, but not significantly higher, than the mean of maximum differences within each individual of 18.9 % ($p = 0.12$). Elevated interdonor variability is consistent with the idea of a continuum of shedders previously proposed to account for observed differences in DNA deposition between individuals [5, 31, 32]. Some donors may shed more cells than others during the sample collection process, but there are not two clear categories of high and low cell content. The variability of cell shedding itself may change between donors, leading to a lack of statistical significance.

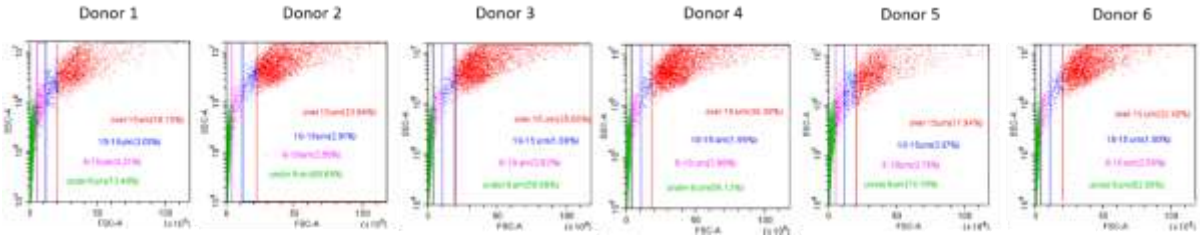

Figure 4. Exemplar FSC v. SSC plots from washed hands rinse collected from 6 separate donors. The first of three replicates is shown. The mean event percentage in each size range was calculated from triplicate analyses and compared to each other donor.

The mean percent of large cells observed in Donor 1's samples differs significantly from the mean percent of large cells observed in all other donors ($p = 0.008$-0.01), except Donor 5 ($p = 0.2$), who showed less consistency than the other donors. Donor 6 also differs significantly from Donor 3 ($p = 0.03$), but not from Donors 2, 4 or 5 ($p = 0.08$-0.5). None of the other mean percentages of large cell events were significantly different from each other in a Student's t-test.

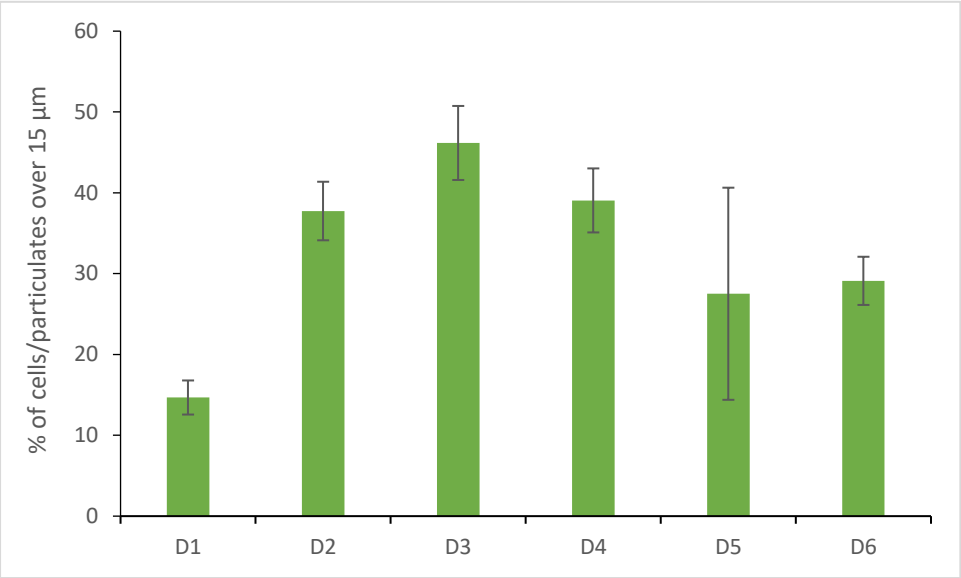

Figure 5. Mean percent of events falling into the large cell category (over 15 µm) from 3 replicates of washed hand rinses from 6 donors. Error bars include 1 standard deviation.

While there is rough consistency between donors, these data tend to support those seen elsewhere documenting individuals' propensity to shed touch DNA as a continuum [5]. Some individuals here deposit more cells than others (e.g. Donor 3 as opposed to Donor 1) (Figure 5), although donors do not fall into clear categories and some show wider variation (Donor 5). Most data previously reported on shedding reflect quantified touch DNA levels, while data here are indicative of cell content. While both DNA levels and cellular proportion of a touch deposit vary between individuals, cells may or may not be the primary DNA source. This is examined more closely in the third experimental phase below.

3.1.3 DNA Localization

After examining the cell distribution patterns above, fluorescent nucleic acid dyes were used in order to locate the DNA within the sample, i.e. to determine which sized cell or fragmentary events contained genetic material. This was determined by separating any particulates or cells whose fluorescent intensity was above the measured autofluorescent intensity of unstained control samples. These events were considered "DNA (+)" as their fluorescence indicated the presence of nucleic acids.

Nucleic acid staining was repeated on all the body fluid sample types, with the hand rinses being of most interest as they are the most informative regarding touch deposit content. Saliva is also of interest as it is expected to contain the full range of potential DNA-sources (i.e. intact nucleated cells, anucleate corneocytes, small leukocytes, free nuclei and degraded cell fragments) and thus serves as an important comparison against the DNA (+) location observed in hand rinses. It was hypothesized that perhaps a small population of nucleated cells could be isolated from the hand rinses by fluorescence and that this might be the significant source of cellular DNA.

Thiazole Orange (TO) stained the nuclei of control cell cultures as expected. As Propidium Iodide (PI) is not permeable in viable/intact cells, it should only stain the nucleic acid of dead or membrane-compromised cells. When both dyes are applied to samples, they should distinguish between live and dead (or intact and compromised) cells as PI will displace the TO in the latter. This was observed in the keratinocyte control samples.

Diamond Dye was examined due to its recent use as a potential in-situ touch DNA detection dye [5]. It was determined not to be well-suited for flow cytometric analysis as it fluoresces broadly across all detection channels (525-780 nm) so would be difficult to use in conjunction with any other dye or

fluorophore. Additionally, it may lack some specificity, as the majority of events across size ranges showed some DNA (+) results not observed with either TO or PI (data not shown).

TO stained the keratinocyte (> 15 µm) population in saliva, as well as much of the small cell and fragment size population (6-15 µm), likely due to the presence of numerous leukocytes. PI staining indicated that the majority of cells were membrane-compromised and contained DNA; this is consistent with dead cells shed inside the mouth which have ceased to function and become detached, but have not broken down to the extent that they have lost their DNA content (Figure 6). The results from TO-stained saliva samples are substantively the same as those from PI-stained samples, indicating that all or most of the shed cells are membrane compromised.

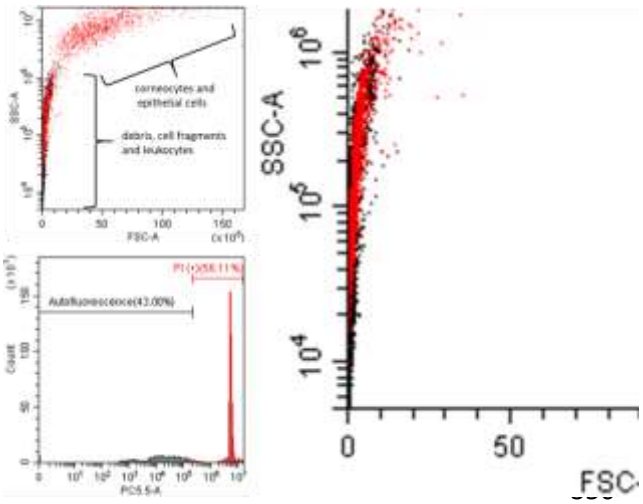

Figure 6. Saliva stained with PI. FSC v. SSC plot (upper left) show keratinocyte clusters (> 15 µm) and leukocyte/debris-sized clusters (< 10 µm). Red indicates nucleic acid fluorescence detected above native autofluorescence levels shown in black (lower left). Close-up (right) of small particulates show DNA (+) events present in saliva, consistent with leukocytes, degraded cells retaining DNA, or free nuclei.

When a similar threshold (shown in red) excluding autofluorescence (shown in black) was applied to the hand rinse samples, their DNA (+) populations were located almost entirely in the large cell-sized region. There were very few events in the small cell or fragmentary size-ranges that stained with PI or TO (Figure 7). This suggests that the prevalence of free nuclei or small DNA-containing cell fragments in hand rinses is limited. As with saliva, TO and PI staining produced very similar results in both washed and unwashed hand rinses, suggesting that the cells deposited from our hands are membrane compromised.

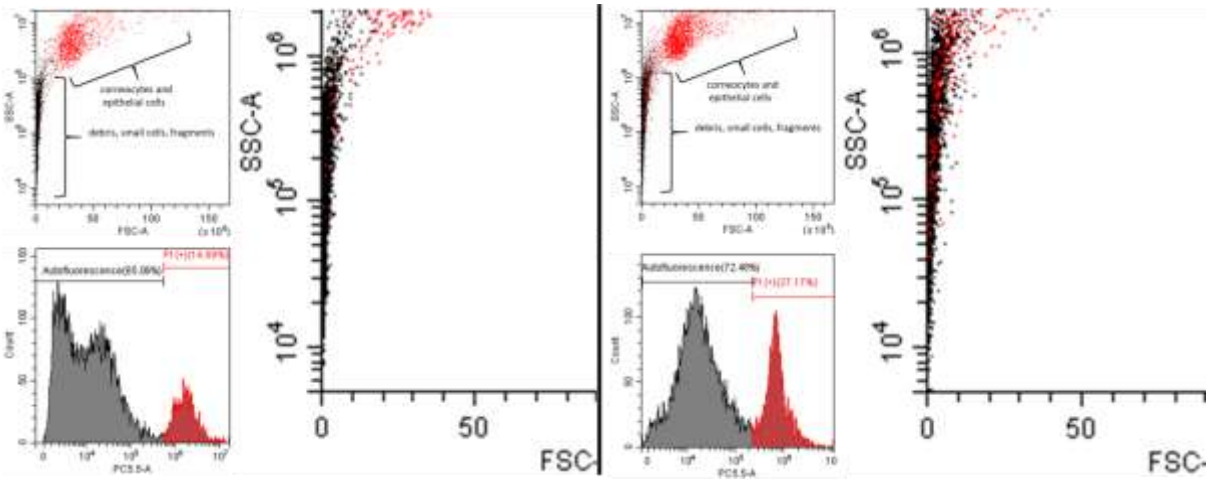

Figure 7. Left: Washed hands rinse stained with PI. FSC v. SSC plot (upper left) show keratinocyte clusters (> 15 µm) and leukocyte/debris-sized clusters (< 10 µm). Red indicates nucleic acid fluorescence detected above native autofluorescence levels shown in black (lower left). Close-up (right) of small particulates shows few DNA (+) events. Right: The same distribution and fluorescence plots for unwashed hands rinse. The close-up (right) shows a limited population of debris

containing nucleic acid, much less than is observable in saliva (Figure 6), although slightly more than seen in washed hands (Figure 7, Left).

Interestingly, similar results were observed in both washed and unwashed hands. Although somewhat more small-sized DNA (+) events appeared visible in unwashed hands than washed hands, the mean percentage of DNA (+) particulates was not significantly different ($p = 0.03$) across donors. This indicates that the biological material accumulated and deposited by hands may not feature many DNA-rich fragments and may, in fact, either be cell-free or consist of intact cells such as anucleate corneocytes.

3.2 Microscopic Examination

Microscopic examination was performed on an aliquot of the stained samples removed just prior to flow cytometry in order to provide visual data correlating with the distribution plots observed. It was intended to establish that the events appearing as DNA (+) keratinocytes or leukocytes based on size and granularity in fact resembled those cell types. It was also intended to explore the nature of the debris staining positive for nucleic acids. Control cells from adherent cultures show clear DNA staining of the nuclei with no fluorescence detected in the surrounding cytoplasmic areas as expected of healthy growing cells.

3.2.1 Cellular Content

The observations of nucleated epithelial cells in saliva was expected since this sample included shed buccal cells. The presence of very small debris and fragments staining positive for nucleic acids in saliva is consistent with the flow cytometry data suggesting the presence of DNA-rich fragments and leukocytes. Likewise, the washed and unwashed hands show very limited debris fluorescence from either flow cytometric or microscopic analysis (with TO and PI, DD is an exception as it appears to fluoresce rather broadly), possibly indicating that these fragmentary fractions yield little contribution to touch deposit DNA. An interesting possibility exposed by these data is that the anucleate corneocytes can be seen clearly lacking nuclei but staining positive for nucleic acid nonetheless.

Very few cells are visible in the nasal lavage and eye wash samples. These samples are notably low cell density, possibly due to their collection as a PBS wash with minimal friction. It is possible that nasal mucosa may not rinse off the nasal cavity easily enough to be collected in large amounts by this sampling. Eye rinse may simply not contain many cells or they may not shed easily. The occasionally observed fluorescence in nasal lavage and eye wash were typically small cell or fragment sized. Very rare corneocytes were observed.

3.2.2 DNA Localization

Washed and unwashed hand rinses display plentiful dully fluorescent anucleate corneocytes, which are also present in saliva and more rarely in eye wash and nasal lavage (Figure 8). Nucleated epithelial cells are fully visible in saliva only. Nasal lavage shows occasional corneocytes and some irregular fluorescence, possibly mucus with diffuse nucleic acids; in two samples, small bright leukocytes were observed with DiamondDye. Eye wash contains very few cells (consistent with low event numbers in flow cytometry) and only rare fluorescent particulates. When fluorescence was seen in eye wash and nasal lavage, it was usually in very small particulates or broad low-intensity smears rather than recognizable cells. Collection methods were not optimized to normalize cell density or characterize each fluid, but to assess potential for contributing to touch deposits on hands. This potential appears lowest in eye wash, followed by nasal lavage and then saliva due simply to DNA (+) particulate density.

Cultured control cells show nuclei only, as expected, since they are healthy and lack degraded DNA diffuse in the cytoplasm which may explain dull fluorescence in shed epithelia and corneocytes.

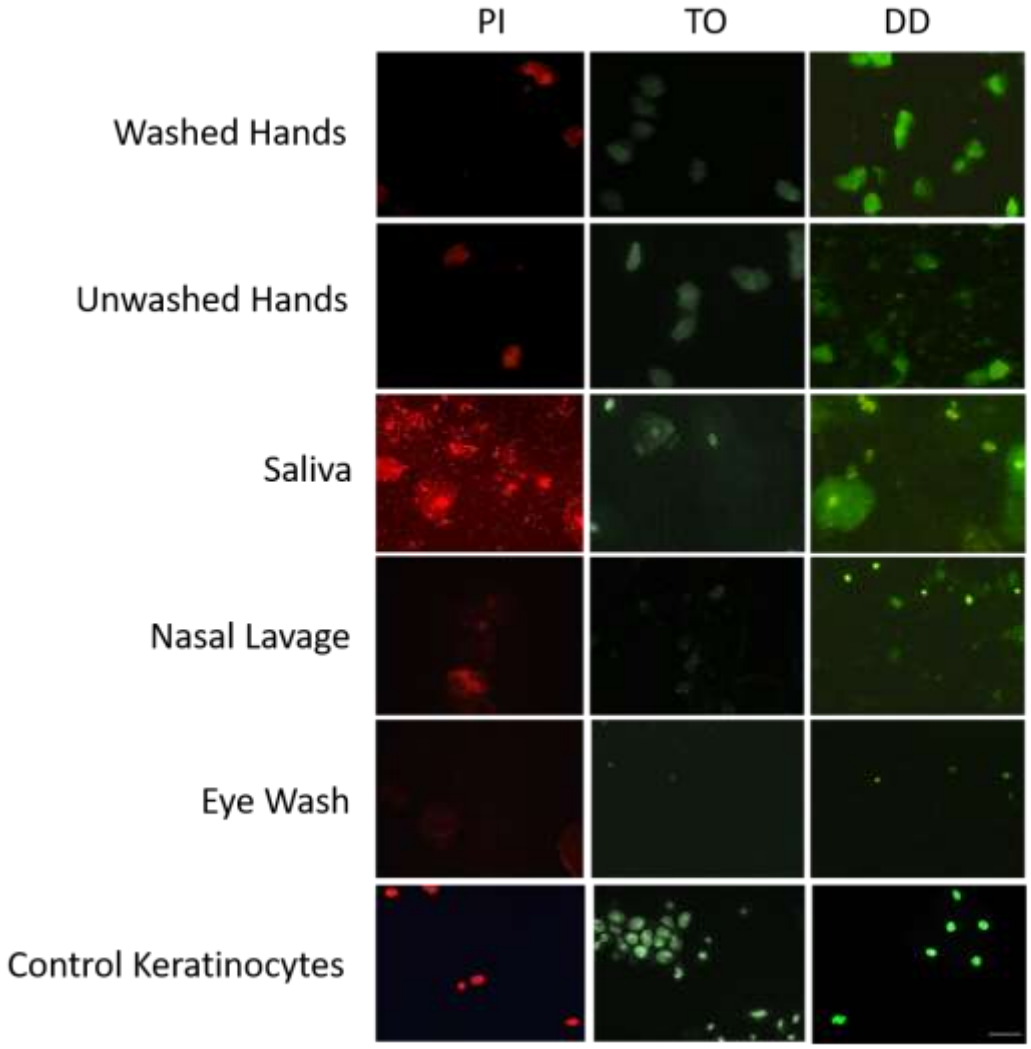

Figure 8. Six sample types (top to bottom: washed hand rinse, unwashed hand rinse, saliva, nasal lavage, eye wash, cultured keratinocytes) stained with three nucleic acid dyes (left to right: propidium iodide, thiazole orange and DiamondDye). Samples examined at 200x. Scale bar represents 30 µm.

The lack of nuclei in any corneocytes observed microscopically in these populations is consistent with published data suggesting no correlation of corneocytes with amplifiable DNA quantity [25]. Yet these anucleate cells do appear positive for DNA with multiple nucleic acid dyes, at a level consistently above autofluorescence. The cornified envelope of shed corneocytes' does not appear to prevent PI staining. Presumably this is because corneocytes are dead and detached and thus their membranes are permeable. Another possibility is that the nucleic acid staining of corneocytes reflects the presence of small, membrane-bound DNA fragments, possibly recruited from cell-free nucleic acids present in sweat [7]. This is consistent with a published report of extracellular DNA bound to corneocyte membranes [6].

These DNA fragments, whether located diffusely within the corneocyte or bound to it externally, are likely too short for standard forensic STR amplification. They may not contribute to current touch DNA casework profiles, which is why the DNA quantities do not correlate with corneocyte cell counts. However, they should be considered as a possible future tool if recovery can be optimized and SNP or sequencing methods more appropriate for short fragments are utilized.

4. Conclusions

Flow cytometry has proved to be a useful tool for the examination of overall trends in cell size and granularity as well as DNA staining in various body fluids and hand rinses. Saliva and unwashed/washed hand rinses displayed large populations of cells, consistent with regular corneocyte shedding. Limited intraindividual variation of cellular content was observed in each sample type, while greater levels of interindividual variability were seen. This supports the theory that some individuals tend to generate and slough more cells than others.

The DNA (+) staining results in corneocytes observed in both flow cytometric data and microscopic examination raise important questions about the nucleic acid content of these anucleate cells. It is possible that the degradation of the nucleus and other organelles (particularly mitochondria which contain DNA) during terminal differentiation leaves residual nucleic acid within the corneocyte. Although such DNA may not be contributing to our current STR typing of “touch deposits,” (if it is too short or inaccessible for amplification), it could prove important with alternate methods of DNA profiling or SNP analysis. This possibility should be explored in future research on the recovery and analysis of corneocyte DNA, as it could be a valuable additional source of touch DNA evidence. The fluorescence data discussed here suggests that although debris makes up the majority of cellular and particulate events in a touch deposit, it does not contain high numbers of DNA-positive fragmentary cells or free nuclei. Although the subset examined microscopically may not have captured every DNA (+) cell fragment, these data suggest debris is making limited contributions to the DNA content of touch deposits. This may allow for size separation or debris removal in touch deposits without risking elevated DNA loss.

Future investigations should focus on isolation and capture of the separate cellular fragments as well as DNA analysis thereof to help determine the origin of not only DNA, but of specifically amplifiable DNA profiles suitable for forensic analysis. Additionally, the DNA content of shed corneocytes should be explored and its potential recovery and value in forensic DNA typing evaluated.

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