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RESEARCH NOTE

Analysis of red autofluorescence (650-670nm) in epidermal cell populations and its potential for distinguishing contributors to 'touch' biological samples [version 1; referees: 2 approved]

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Abstract

Interpretation of touch DNA mixtures poses a significant challenge for forensic caseworking laboratories. Front end techniques that facilitate separation of contributor cell populations before DNA extraction are a way to circumvent this problem. The goal of this study was to survey intrinsic fluorescence of epidermal cells collected from touch surfaces and investigate whether this property could potentially be used to discriminate between contributor cell populations in a biological mixture. Analysis of red autofluorescence (650-670nm) showed that some contributors could be distinguished on this basis. Variation was also observed between autofluorescence profiles of epidermal cell populations from a single contributor sampled on different days. This dataset suggests that red autofluorescence may be a useful marker for identifying distinct cell populations in some mixtures. Future efforts should continue to investigate the extrinsic or intrinsic factors contributing to this signature, and to identify additional biomarkers that could complement this system.

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Referee Status: ✓ ✓

	Invited Referees	
	1	**2**
version 1 published 16 Feb 2016	✓ report	✓ report

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Introduction

The difficulties associated with interpreting complex DNA mixtures are well known in the forensic community, and are becoming more prevalent with the sharp increase in 'touch' or trace samples among forensic laboratories' caseloads[1]. Differentiating cell populations from individual contributors in a biological mixture before DNA analysis is a potential way to overcome this issue. While strategies exist to selectively label cell populations from distinct contributors based on their immunochemistry and then physically isolate cells from the mixture prior to DNA profiling[2–4], there is a dearth of studies demonstrating cell separation techniques on touch samples. This is likely due to the fact that cell populations in these samples mostly, if not entirely, consist of fully differentiated keratinocytes which have limited reactivity to common molecular probes used to target surface antigens[5,6].

An alternative approach is to avoid the need for probe binding by harnessing the intrinsic fluorescence of compounds found in or on epidermal cells. Here we report on our analysis of autofluorescence in the red region of the spectrum (650–670nm) of epidermal cells collected from surfaces touched by seven different individuals across multiple days, and the implications this may have for processing complex biological mixtures in forensic casework.

Methods

Touch samples were collected from seven volunteers using the following protocol which was approved by the VCU-IRB (#HM20000454_CR). Volunteers rubbed a sterile polypropylene conical tube (P/N 229421; Celltreat Scientific) for five minutes using their entire hand (i.e., palm and fingers). Cells were collected from the surface with six sterile pre-wetted swabs (P/N 22037924; Fisher Scientific) followed by two dry swabs. To elute the cells into solution, the swabs were manually stirred then vortexed for 15 seconds in 10 mL of ultrapure water (18.2 MΩ·cm). The entire solution was then passed through a 100 μm filter mesh prior to flow cytometry. Flow cytometry analysis of eluted cells was performed on the BD FACSCanto™ II Analyzer (Becton Dickinson) equipped with 488 nm and 633 nm lasers and a 660/20 nm detector filter. Channel voltages were set as follows: Forward Scatter (FSC, 150V), Side Scatter (SSC, 200V) and Allophycocyanin (APC, 250V). FSC and SSC channels were used to gate intact corneocytes for subsequent autofluorescence analysis. Gating of cell populations and generation of histogram profiles for each contributor was performed using FCS Express 4.0 Flow Research Edition (De Novo Software, Inc.).

Results and discussion

Dataset 1. Flow cytometry source data for individual contributors

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Flow Cytometry Standard (.fcs) format files are labeled by the corresponding panel in Figure 1 and the Donor ID.

Fluorescence histograms of individual cell populations from different donors are shown in Figure 1. For ease of comparison and visualization, profiles have been overlayed and grouped by the day

on which cells were deposited, collected, and analyzed by flow cytometry. Clear differences in the red fluorescence (APC) channel are observed between several pairs of donor cell populations, particularly J16-D02 during the first experiment and J16-S07 in the second experiment (Figures 1a and 1b respectively; Table 1). Most experiments resulted in one or more contributor cell population(s) whose fluorescence profile(s) could be distinguished from the others collected that day, such that a fluorescence intensity gate could be designed that would be expected to capture that contributor's cells to the exclusion of (or minimal contribution of) cells from other contributors. However, significant and/or complete overlap was observed between many donor pairs (e.g., A42-B17 in Figure 1a; I66-S07 in Figure 1d). Sometimes, overlap of fluorescence distributions was such that gating could potentially separate the contributors into two or more groups (e.g. Figure 1d: A42, B17, I66, R12 and S07 in one group; D02 and J16 in another group). All contributors from the final experiment exhibited overlapping fluorescence histograms (Figure 1e).

Cell populations from J16 and D02 showed a great deal of disparity in fluorescence intensity in the first experiment, such that overlap between these populations was minimal (Figure 1a). There was somewhat less distinction – and thus more overlap – observed between the same contributors during a second replicate (Figure 1c); during a third, overlap between the two populations was substantial (Figure 1d). As these results suggest, fluorescence intensity values for cell populations derived from any given contributor varied in distribution across replicate experiments on different days. Figure 1f shows overlayed histograms for J16 cell populations; mean fluorescence intensity values ranged from 589 to 2606 relative fluorescence units (RFUs) across five sampling days (Table 1).

The underlying cause of red autofluorescence in these epidermal cell samples is currently unclear. Cells deposited through touch are likely primarily derived from the outermost epidermal layer (stratum corneum) which can contain a number of fluorescent compounds including tryptophan and tyrosine[7,8], melanin, keratins, NADH and flavins[9], lipofuscins[10], and porphyrins and porphyrin precursors[11,12]. However, many of the corresponding emission maxima for these molecules occur at shorter wavelengths than what was examined in this study (e.g., amino acids, keratin, NADH, all have maxima below 550nm[9]). Porphyrin molecules exhibit emission maxima between 630–680nm[11]. Their abundance within the epidermis may be influenced by bacteria on the skin that produce porphyrin molecules with similar fluorescence emission profiles[13]. Exogenous sources such as plasticides[14] or other biological compounds (e.g., chlorophyll[15]) may also produce fluorescence, and could potentially be transferred to donors' hands and subsequently to the tube surface (with cells) through touch or contact.

Regardless of the ultimate source for the observed differences in cell population fluorescence, this initial data set indicates that autofluorescence may be a useful marker for distinguishing between cell populations in a mixture. The non-destructive nature of flow analysis and the fact that autofluorescence monitoring does not require special reagents beyond those maintained in any laboratory (e.g. no probes required) are advantages when considering their potential front-end use in forensic analyses.
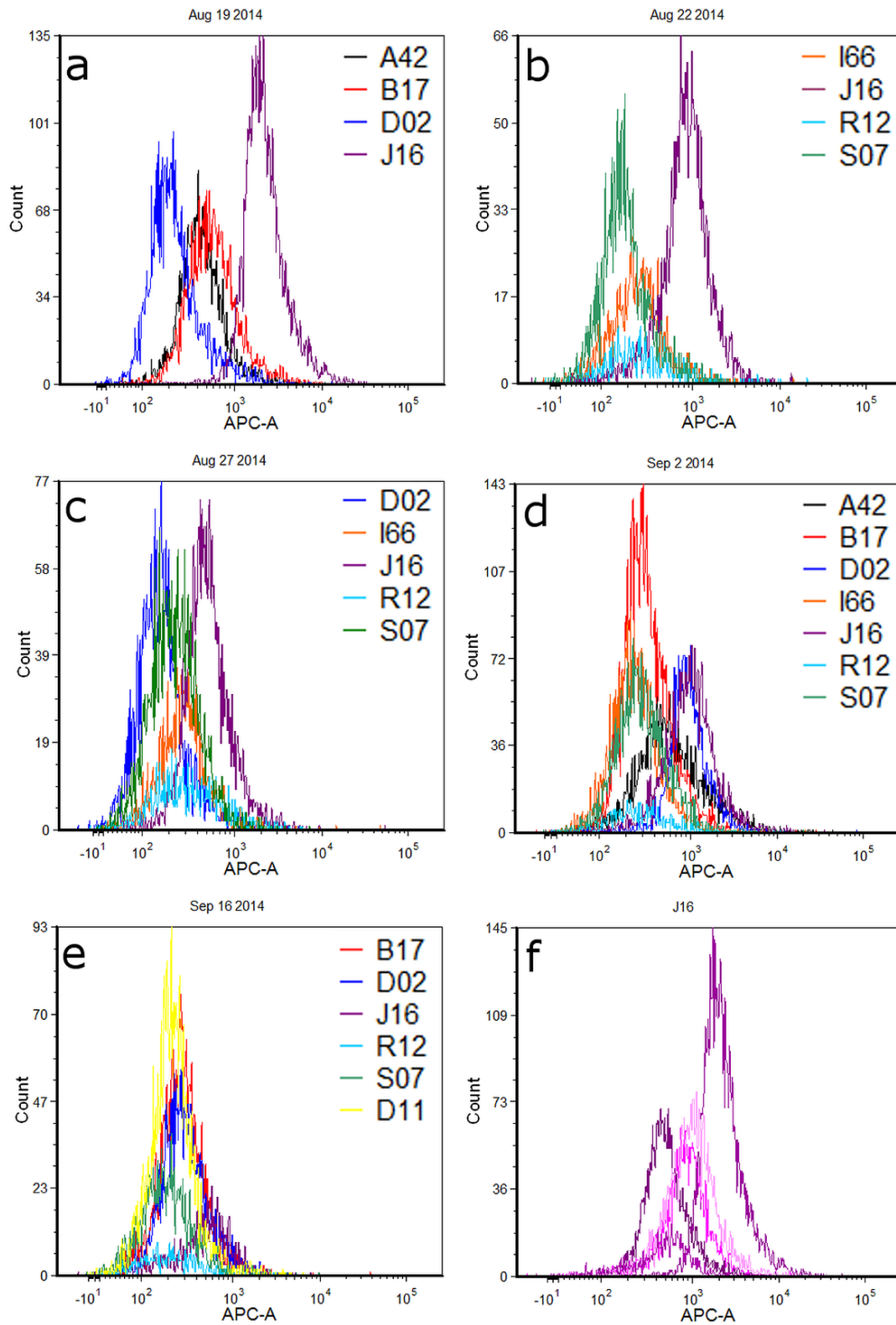
Figure 1. Overlayed red fluorescence channel histograms for epidermal cell populations from touch samples. Panels **a**–**e** show different combinations of donors cell populations each sampled and analyzed on the same day. Figure 1f is a histogram overlay of cell populations from contributor J16 across five different experiments.

Table 1. Fluorescence histogram statistics for contributor cell populations[1].

Fig 1a					Fig 1b			
Donor	Mean	Median	# Events[2]		Donor	Mean	Median	# Events
A42	540	427	3903		I66	341	253	1573
B17	743	556	4625		J16	996	842	3375
D02	305	212	5158		R12	497	252	599
J16	2606	2024	6475		S07	236	177	2497
Fig 1c					**Fig 1d**			
Donor	Mean	Median	# Events		Donor	Mean	Median	# Events
D02	208	160	3653		A42	959	554	4320
I66	372	276	1983		B17	409	307	7727
J16	635	491	3767		D02	1114	907	3524
R12	469	298	1090		I66	314	244	5014
S07	279	226	3751		J16	1245	982	4702
					R12	457	260	861
					S07	376	277	4676
Fig 1e					**Fig 1f**			
Donor	Mean	Median	# Events		Donor	Mean	Median	# Events
B17	349	280	3665		J16a	2606	2024	6475
D02	362	287	3041		J16b	635	491	3767
J16	589	515	1156		J16c	589	515	1156
R12	302	208	493		J16d	996	842	3375
S07	259	190	2028		J16e	1245	982	4702
D11	276	220	4230					

[1]Data is organized according to the histogram overlays shown in Figure 1. Mean (arithmetic) and median values are in relative fluorescent units (RFUs).
[2]Flow cytometry cell 'events' correspond to populations within FSC and SSC gates that select for intact epidermal cells.

The variation across multiple samples from the same donor suggests that the level of autofluorescence is likely not a unique or identifying feature for a particular individual. However, to be of use in separating components of a biological mixture, a feature need not be unique; it simply needs to be distinctive among the contributors to that particular mixture. The ability to separate out even one contributor (or to separate a mixture of four contributors into two mixtures of two) may render the remaining mixture more interpretable in downstream DNA analysis. Further, the possibility that some combination of endogenous and/or exogenous factors could impart distinct optical properties to contributor cell populations in a particular mixture sample warrants further exploration.

Future efforts will continue to focus on isolating the molecule(s) responsible for fluorescent differences in touch epidermal cells through a combination of targeted immunofluorescent assays, chemical characterizations, and complex spectral analysis of autofluorescent profiles. Additionally, we are working on using optical signatures such as these to facilitate physical isolation of epidermal cell populations using flow cytometry-based strategies such as fluorescent activated cell sorting (FACS) for the purposes of generating single source genetic profiles from touch mixtures. Although previous work suggests that analyzing DNA profiles directly from isolated epidermal cells may be a challenge due to the prevalence of extracellular or 'cell-free' DNA in touch samples[16], the sheer quantity of cells that may be recovered from these sample types (up to $\sim 1 \times 10^{5}$,[16]) may help to overcome such obstacles.

Data availability

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    Data Source

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Current Referee Status: ✔ ✔

✔ **Mark Perlin**
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The article's title and abstract are appropriate. The design, methods and analysis have been explained, and are appropriate for this preliminary report. The conclusions are supported by the results. Data have been provided. Some context and suggestions for analysis are provided here.

Separation of cellular genetic material is central to forensic DNA analysis. Separation stages include:
1. Cells. Labs *separate* cells out of mixed cellular populations based on physical characteristics. For example, differential extraction can enrich sperm in one cell fraction relative to epithelial cells in another fraction. The red autofluorescence described in the paper occurs at this front-end stage.

2. DNA. Extraction procedures *separate* nucleic acids from other molecules. Such procedures include organic, non-organic, chelex, FTA or silica methods.

3. Loci. PCR *separates* short DNA regions from the rest of the genome by amplifying (i.e., purifying) these specific regions relative to background sequence.

4. Fragments. Fluorescent electrophoresis *separates* DNA fragments by size, identifying length polymorphism alleles and determining their relative quantity.

5. Genotype. Computer *separation* of electrophoretic data can determine the genotypes of each contributor to a DNA mixture[1]. Back-end methods that do not separate contributors[2] have limited applicability for resolving complex DNA mixtures.

This paper focuses on the first step, front-end separation of mixed epithelial cell populations. The authors investigate a novel physical assay – red autofluorescence in the 650-670 nm range. Their preliminary results demonstrate effective separation, with some cell populations clearly distinguished from others based on fluorescent frequency.

There are some limitations to the current procedure. The partial autofluorescence separation is incomplete, and can vary for the same subject between samplings. However, as the authors note, "the ability to separate out even one contributor [subset] may render the remaining mixture more interpretable in downstream DNA analysis." This ability, coupled with automatic fluorescent activated cell sorting (FACS) and automatic DNA mixture data analysis (e.g., TrueAllele® computing), could be quite powerful.

Joint Bayesian analysis of multiple STR samplings of low-level DNA mixtures can recover considerable

identification information[3]. The autofluorescence method reported here could produce analogous data by taking FACS samplings at different fluorescent frequencies. Joint computer analysis of these different frequency-sampled STR amplifications would then complete the (mathematical) mixture separation into informative (probabilistic) genotypes.

The authors propose a novel front-end DNA separation method. As with most front-end methods, the cell separation is incomplete. However, their autofluorescence separation can become more complete if used in conjunction with other front-end discrimination methods. Moreover, combining their front-end cell separation with available back-end computer data separation[1,3] could extract considerable identification information from complex DNA mixtures.

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Competing Interests: No competing interests were disclosed.

Referee Report 28 October 2016

✔ **Timothy J Verdon**
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The authors have investigated skin cell analysis by FACS in forensic biological analysis, and present some results which shed some light on the potential, or lack thereof, for this kind of technique. The experiment is technically sound and well presented, and although there is no statistical weight placed on the differences or similarities among cell species, some trends are obvious.

Some minor amendments and areas for future examination would improve the paper:
- It must be noted earlier on (i.e. at the end of the first introductory paragraph) that much of the DNA in touch samples will be exogenous - the use of cell sorting for these kinds of samples may not actually yield appropriate quantities of DNA for profiling.

- The methodology of collecting touch samples raises some questions - the swab type used may have an effect on the cell structure and elution of the sample, possibly even may affect / introduce autofluorescence in some cases. Also were the swabs dried post-sampling or eluted straight afterwards? In actual casework this may also affect the quality of the keratinocytes, as will the lag between sampling and analysis. It would be of benefit to repeat this study with older samples,

perhaps placed onto a swab and dried for a week before analysis, compared to pristine cells collected via a glass bead method[1] without the need for swabs at all.

- The cells collected and analysed herein may not all be borne of the individual who touched the item - how controlled were the volunteers before touching? Did they have to wash their hands? Did they have contact with others or other work environments? The only way to assess this fully would be to examine the profiles from these samples post-analysis.

- Following this, the samples may not actually yield sufficient DNA, as rightly pointed out in the discussion. It seems a shame that these samples were not separated and profiled to examine this further.

Overall, the publication of this type of data should be encouraged, and hopefully more thorough experiments of this kind will follow on from this work.

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I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.