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PRE-EXTRACTION SEPARATION OF MIXED SOURCE DNA SAMPLES THROUGH MICROMANIPULATION AND DIRECT PCR SHORT TANDEM REPEAT TYPING OF CELLS.

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ABSTRACT

Highly sensitive DNA analysis methods have been in-use for forensic casework at the NYC Office of Chief Medical Examiner since January 2006. Having pioneered these techniques in the United States, we are the first American lab to use these methods to provide accurate and reliable forensic DNA testing results for evidence containing relatively small amounts of DNA. These items are typically touched or grabbed such as the handles of weapons, tools used to gain access to a safe for example, and clothing with no apparent biological staining. This evidence is recovered in the investigation of a wide variety of criminal offenses including homicide, sexual assault, felony assault, and property crime cases. As the evidence is often touched by multiple persons, not just one perpetrator but perhaps multiple perpetrators and/or the victim(s), often the results include DNA contributions from multiple individuals.

Deconvoluting these mixtures, particularly when dealing with initially small amounts of DNA, 100 pg or less, requires care and may be a complex process. Sometimes, the individual DNA profiles of the contributors cannot be determined, and even when they can, these profiles may be consistent with the victim and thus, may not be informative. In fact, from our extensive experience analyzing touched items, in approximately 60% of samples, no foreign DNA profiles were found.

This study examined several approaches in order to deconvolute mixed DNA samples, including micromanipulation techniques that use microscopy often in conjunction with cellular staining to separate individual cells, before they become inadvertently pooled in a DNA extraction.

Research on collecting single cells showed that spheres made of Arabic gum/glycerol were best for lifting individual epithelial and sperm cells. Fingerprints, on the other hand, should be swabbed using cotton threads for entire or spheres of rubber cement for partial prints. If prints are located on lager objects that cannot be examined under the microscope, they can be lifted using SIRCHIE tape and either cut or swabbed for DNA extraction. Arabic

gum/glycerol, rubber cement, cotton, and SIRCHIE tape do not inhibit PCR reactions, the downstream method to generate STR profiles.

Using a few cells or fingerprints may lead to partial STR profiles, but goal of this study was to obtain database eligible profiles. These are AmpF*l*STR® Identifiler® PCR (Life Technologies, Foster City, CA) profiles that are at least to 70% complete. In order to obtain high fraction profiles, swabs of fingerprints and epithelial cells were treated with ZyGem (prepGem® tissue extraction kit, Zygem, New Zealand), a direct DNA extraction procedure that features a modified enzyme, which works at high temperatures followed by AmpF*l*STR® Identifiler® using 31 cycles. Sperm cells needed treatment with DTT to open the cells and release the DNA.

Further examination of fingerprints revealed that over time less DNA can be recovered and that the substrate is relevant for the recovery. Metal may lead to DNA degradation, while glass showed highest fraction profiles.

A three-person mixture study on fingerprints showed that sectioning and independent processing led to a few single source profiles and many mixtures of which a major contributor could easily be deduced.

The Axiozoom, a high resolution stereomicroscope, is well suited to aid in collecting fingerprints as well as single cells. Testing 10 epithelial and 10 sperm cells showed database eligible profiles. As shown for fingerprint mixtures, sectioning of samples, e.g. making several cell collections and their independent processing could help to solve complex mixtures.

(561 words)

EXECUTIVE SUMMARY

1. Introduction

The OCME routinely examines touched items that were recovered from crimes, including homicides, felony assault, sexual assault, robberies, and burglaries. These items may include handled objects, such as weapons or tools that were touched by multiple persons. Therefore, DNA recovered from these samples typically originates from two or more individuals. In some cases, mixtures can be identified due to morphological differences, if examined under the microscope, such as in sexual assault evidence, which is typically comprised of sperm and vaginal epithelial cells.

Micromanipulation involves techniques to separate sample mixtures to individual contributors. These techniques utilize microscopes to make the cellular material visible, often in conjunction with cell-staining and the use of tools to physically separate and collect single cells. The separation is done before the cellular material from different contributors become inadvertently pooled in DNA extraction.

The use of a single-cell typing approach to analyze single-cell profiles would by definition eliminate the need to use additional methods to deconvolute a DNA mixture. However, this approach may lead to partial profiles that lack completeness to compare them to databases. In this study, AmpF*l*STR® Identifiler® PCR Amplification Kits were used that amplify 15 autosomal loci plus Amelogenin, for a maximum of 30 autosomal alleles per donor. Profiles that were at least to 70% complete were considered valuable, since such profiles can be used to search databases. Consequently, few cells were collected and processed. The cellular material used in this study included fingerprints, epithelial (buccal), and sperm cells.

2. Collection of Single Cells

Various tools were tested to collect single cells from a mixture of cells, including microcarriers, tape, and needles as well as a staining procedure that stains male and female cells differently (X/Y FISH).

2.1 Microcarriers

Microcarriers are tiny spheres that can attach to a selected cell. They are held by tweezers, whereby the correct position is controlled with a mirror to ensure that the sphere sticks out and can be used to touch the cell. Microcarriers hold the cell with greater force than the cell holds on to the surface. Thereby single cells can be collected and transferred into a reaction tube.

Commercially available microglobes (SoloHill, Cytodex™3), coated with collagen, ProNectin F, and CT (proprietary), were tested. The microglobes from SoloHill were easy to handle but were not adhesive for lifting single cells and therefore were excluded. The Cytodex™3 microglobes were soft and often deformed when held with tweezers. Their adhesiveness was a little better compared to the SoloHill microglobes. However, the Cytodex™3 microglobes could be used to pick up skin flakes but were not useful for lifting epithelial (buccal) or sperm cells. In addition, it was found that the Cytodex™3 microglobes inhibited PCR reactions and were consequently excluded.

Further, various glues were tested for lifting single cells: aureka® glue was not sticky to pick up single cells. Pritt, an office glue, was sticky enough to lift single cells but stuck to the needle and could not easily transferred into the reaction tube. Both glues were excluded.

Arabic gum/glycerol could be formed to tiny spheres that could be hold by a tungsten needle. These spheres could be used to pick up single cells and were transferrable into a reaction tube. Arabic gum/glycerol showed no PCR inhibition. Spheres made from rubber cement could be hold by tweezers and were usable to swab tiny areas. Rubber cement also showed no PCR inhibition. Arabic gum/glycerol was used to collect single cells (buccal, sperms and skin flakes), while rubber cement was used to swab tiny areas (partial fingerprints).

2.2 Tape

If prints or cells are located on larger objects that are too large to put under a microscope, such as doorframes or walls, they may be transferred by tape, followed by cutting or swabbing and processing for DNA recovery.

Several forensic (Remco, DIFF-lift, and SIRCHIE) and office (tesa and Scotch) tapes were tested. Thin knifes as the X-Acto knife and the feather blade 5300 were used to cut the tapes. Since Remco is structured and DIFF-lift is thick, areas containing single cells could not easily be cut out and were excluded. SIRCHIE and tesa were suitable for manual cutting and did not show any PCR inhibition. Therefore further testing was performed using SIRCHIE, a forensic tape.

2.3 Needles

Hydrodermic and tungsten needles could be used to scrape cells from a smooth surface. However, the tungsten needle was impractical to pick up single cells and the hydrodermic needle scraped too many cells from surfaces. Therefore the tungsten needle was only used to hold glue spheres that were used to lift and transfer single cells.

2.4 X/Y FISH

If male and female cells cannot be distinguished by shape (sperm and epithelial cells) they could be stained differently to facilitate their physical separation. Fluorescent *in-situ* hybridization (FISH), which stains the X and Y chromosomes orange and green, respectively, was applied. The protocol was adjusted to be used on the PALM, utilizing membrane (PET) slides.

3. Protocol for DNA extraction and STR amplification of fingerprints

Fingerprints can be an important but unreliable source of DNA for forensic testing. They can yield in little to no DNA or result in DNA profiles that are

suitable for upload to forensic STR databases. Over 700 fingerprints were processed to find the best procedure for DNA extraction and amplification in order to obtain STR profiles of a quality that can be used to search databases.

3.1 DNA extraction from fingerprints

A total of 756 fingerprints were processed for this study. For DNA extraction, when expecting low amounts, direct PCR or DNA extraction methods performed in one reaction tube have several advantages: (i) DNA could not get lost by transferring the solution into other reaction tubes, (ii) DNA would not be shared or fragmented through additional pipetting steps, and (iii) possibilities of contamination would be low, because the tube is kept closed until aliquots are taken for quantification or STR amplification. A disadvantage of this approach is that the DNA solution may not be pure and could contain PCR inhibitors.

Three extraction methods, two direct extraction (one-tube, developed in-house, and ZyGem, commercially available) and one multistep extraction (HighSense, developed in-house for touched items, which includes purification and concentration steps) protocols were compared.

The highest DNA recovery was obtained by using the HighSens DNA extraction, followed by Zygem and the one-tube method.

3.2 STR profiles from fingerprints

Following DNA extraction, the fingerprint samples were amplified using AmpF*l*STR® Identifiler® PCR Amplification Kits that amplify 15 autosomal loci plus Amelogenin. Identifiler® (ID) was utilized by applying the recommended 28 cycles and, in order to improve the outcome, with 31 cycles. In addition, AmpF*l*STR® Identifiler® Plus (ID+) was tested, which was developed to overcome PCR inhibition. ID+ was applied by using the recommended 32 cycles of amplification.

Most STR profiles obtained from fingerprints were partial (between 1-99%). Profiles that were at least to 70% complete were considered as database eligible.

Of 756 fingerprint samples 46 mixtures (6%) were detected by additional signals at multiple loci. These mixtures were excluded from further analysis, leaving 710 single source samples.

For an independent comparison, only samples that measured at least 2 pg/µl of DNA concentration were compared, which included 436 samples. Of the samples that were below this concentration (n=274), only 21 resulted in profiles that were at least 70% complete.

For this comparison the one-tube method followed by ID31 amplification was used as baseline. Regarding DNA extraction, using ZyGem increased the probability of obtaining database eligible profiles, while HighSense extraction led to a decrease. Regarding STR amplification, using ID+ increased the outcome, while ID28 decreased it. However, this effect could not be seen for ZyGem and HighSense.

The best protocol for DNA extraction and STR amplification of fingerprints based on this study was ZyGem followed by ID31.

4. Fingerprint analysis

When an object is handled, cellular material and DNA may be deposited. Touched objects such as weapons, tools or door handles can be used as evidence in a wide variety of criminal offenses. In order to have standardized conditions, volunteers were asked to refrain from washing their hands at least 2 h prior to donating a fingerprint.

4.1 Partial fingerprints

In order to deconvolute mixed samples, items that were touched by multiple persons, individual cell clumps could be collected. DNA was extracted from five clumps/flakes and STR profiles were generated using ID31. The profiles obtained were below 30% and therefore not database eligible. Then, a small area of a fingerprint was swabbed. The resulting profiles showed a broader variation but were still below 70% completeness.

From these results it was concluded that fingerprints should be swabbed in their entirety, in order to reach database eligible profiles.

4.2 Visible deposition and fraction profile

Fingerprints vary greatly in their density of deposition. A shedding score (1 to 5) was assigned to describe the deposition of under the microscope visible particles. A lower number reflected sparse and a higher number dense shedding. Over 100 fingerprints were assigned per volunteer. A comparison of four volunteers showed that individuals could be considered as good or not so good shedders. However, when those fingerprints were further processed (DNA extraction and STR amplification), it was shown that good shedders could lead to not so good fraction profiles, while bad shedders could result in better profiles. Therefore, the shedding score was not considered as a reliable predictor for profiling success.

4.3 Left versus right fingerprints

Handedness determines which hand is more often used. Right handed individuals use their right hand more often and left handed individuals their left hand. It was tested whether the dominant hand deposits more cellular material that contributes to the STR profile. The thumb-prints of three right handed volunteers were tested and showed that the dominant hand resulted in slightly lower fraction profiles. This outcome may lead to the suggestion that the dominant hand is exposed to some abrasion while being used and therefore left less material was deposited onto the glass slide. In contrast, the dominant hand is also used for touching the face, or other areas that contain sebaceous glands, thereby with a certain possibility transferring extracellular DNA.

4.4 Fingerprints on various substrates

Various substrates were tested to find out if they affect the cellular material of fingerprints by generating STR profiles. This knowledge may be useful for

casework in order to swab the areas that are most likely to lead to database eligible profiles. The substrates tested included, glass, metal (a cleaned US Quarter dollar made of Cu and Ni) paper and plastic (sheet protector made of polypropylene). The highest fraction profiles were obtained from glass, followed by plastic and paper, while no profiles were obtained from the US Quarter dollar.

4.5 Time course

Time and environment may have an effect of the qualities and quantities of DNA recovered from fingerprints. A time course study was performed and over time fingerprints resulted in lower fraction profiles. This effect was significant after 10 days. Nevertheless, full STR profiles were obtained after 40 of storage at room temperature. However, for best outcomes, touched items should be processed at the earliest convenience.

4.6 Three person mixtures

Routinely used methods for sampling evidence from violent crimes, such as weapons, tool or door handles could be touched by multiple persons, often leading to DNA mixtures of which no individual contributor can be determined. By sectioning this evidence into segments that may cover at least a single fingerprint and processing these separately may improve the outcome of STR profiles, showing a major and minor contributor or even by obtaining single source profiles.

The trunk of a cleaned beer bottle was sectioned into 6 segments. 3 persons were allowed to touch the bottle. The sections were swabbed and processed independently.

It was found that the last person who touched the bottle did not mainly contribute to the STR profiles obtained. Therefore, it could be assumed that the order of which the bottle was touched was not relevant. Further, this sampling method, of which six samples per bottle were processed, led to some single source and

deducible profiles that were more than 70% complete (database eligible), thereby revealing an immense value of this approach for forensic applications.

5. Micromanipulation

Micromanipulation is the separation of mixed cellular material prior to DNA extraction and STR amplification. It includes the use of a microscope and physical manipulation, often done in conjunction with staining. Three instruments were tested and compared: (i) PALM (Positioning and Ablation with Laser Microbeams), an inverted microscope, which utilizes laser energy to transfer areas or cells of interest into the lid of a reaction tube, (ii) Axiozoom, a high-resolution stereomicroscope, and (iii) aureka®, a robotic arm that can be used with the Axiozoom for micromanipulation. These instruments were tested on fingerprints, epithelial (buccal), and sperm cells for their efficiency to aid in collecting single cells.

Since the PALM can only be used for cell picking by selecting an area of interest, cutting through the membrane and catapulting this area into a reaction tube, it cannot be used for swabbing fingerprints. The Axiozoom is suitable for swabbing fingerprints either with manually operated tweezers that hold small spheres of rubber cement or with the robotic arm aureka®. The use of aureka® however is better suited for smaller areas (partial fingerprints).

Single epithelial (buccal) and sperm cells can be collected by using the PALM, or the Axiozoom either operated with the aureka® or manually. However, the fraction profiles of 10 buccal cells were significantly lower for the PALM than for the Axiozoom (aureka® or manually). Using the Axiozoom for cell collection, most of the STR profiles were database eligible (>70%). The outcome was similar for 10 sperm cells, but their fraction profiles were higher for all instruments tested. That could be explained by their cell walls, which enclose sperm cells and make them easier to collect.

6. Implications for policy and practice

Interpretation of admixed STR profiles from multiple donors represents a challenge for forensic laboratories. In particular, when dealing with initially small or trace amounts of cellular items, such as in touched objects, which require care and may be complex. This study approached this challenge by testing methods to collect single cells as well as cellular material of fingerprints using instruments appropriate for micromanipulation, by sectioning samples and processing them independently, and by testing protocols to extract DNA and amplify STR loci in order to generate database eligible profiles.

7. Implications for future research

Generating STR profiles from fingerprints is challenging due to the fact that very little cellular material was left with the print. Therefore, this study analyzed an enormous number of fingerprints. Still it would be interesting if other laboratories could confirm the outcomes that were generated by this study. This could help to advance to more standardized protocols for handling fingerprints in forensic casework.

Validating the Axiozoom for forensic casework could be beneficial, since this instrument can be used on a wide variety of criminal cases, including property crime, and sexual assault cases, where mixed samples of two or more contributors may be deduced.

Further research testing more FISH probes for separating cellular mixtures may facilitate single cell collection of different contributors.

8. Dissemination of research findings

This work resulted in one publication, one will be soon submitted and an additional one is planned. Findings were presented on three meetings (oral presentations) as well as on several in-house educational seminars for approximate 150 criminalists at the OCME.

(2,562 words)

FINAL REPORT

1. Introduction

1.1 Statement of the Problem

Over the five years that the OCME has routinely examined touched items, more than 50,000 samples were tested for STR profiles. These items were recovered from crimes ranging from homicides to felony assaults, sexual assaults, robberies and burglaries. When an object is handled, cellular material and DNA is deposited. Since touched objects such as restraints, weapons, tools, (e.g., screwdrivers), or door handles are commonly handled by multiple persons, the DNA recovered from these samples typically originates from two or more individuals. Although approximately 45% percent of the mixtures detected may be deconvoluted to yield a major profile, this profile is often, 29% of the time, consistent with the victim rendering these mixtures at best only suitable for comparison to the minor component. For other mixtures, none of the DNA profiles of the individual contributors may be resolved and the mixtures are also only suitable for direct comparison. In order to make these comparisons, the suspect must be identified through non-DNA means which does not always occur. For the remaining mixtures, there may be a lack of sufficient amount or quality of STR data to draw conclusions. Taking these factors into consideration, approximately 60 percent of STR typing results from touched item samples do not yield foreign DNA profiles. These results demonstrate that protocols that isolate foreign DNA or at least prevent irresolvable mixtures and enrich low level foreign contributors are warranted.

1.2 Literature Citations and Review

In recent years, the focus of forensic DNA evidence collection and analysis has shifted from the macroscopic to the microscopic level. STR profiles are routinely obtained from items without biological staining visible with the unaided eye (*1*). This shift has been facilitated by technological improvements in sample collection, extraction, amplification and separation leading to the generation of an

STR profile from materials that have merely been touched by an individual or individuals (reviewed in *2*). Using highly optimized DNA collection and analysis methods, database-eligible STR profiles may be generated of the equivalent of 3 diploid cells (*3-6*). This level of sensitivity has lead to the routine acceptance of touched evidence items for STR analysis within our laboratory.

DNA deposited on touched items most likely originates from skin. Skin is the largest organ of the body, and undergoes constant proliferation and renewal. As the basal cell layers mature, they progress to the upper epidermal layer. During the maturation process genome DNA of these cells become fragmented through an apoptotic process, and the cytoplasm condenses and becomes highly keratinized (*7*). Thousands of these cells are shed and transferred onto items the skin comes in contact with daily (*8, 9*). A majority of cells shed from the upper layer of the epidermis are non-nucleated following maturation, however, some nucleated cells, possibly from lower granular layers, can be visualized from fingerprints (*1*). Staining of skin tissue as well as cotton swabs commonly used in evidence collection has shown that the outermost layers contain degraded, single stranded DNA. High molecular weight DNA can be extracted from tape lifts and swabs taken from the necks of volunteers, indicating that even though a large portion of cells deposited through touch or shedding do not contain nuclei, DNA capable of producing a forensically useful STR profile is present (*10*). The type of contact and amount of time needed for a significant transfer of DNA has been researched by many groups (*11*). Routine contact with an item, simply by being held or used for a period of as little as 10 seconds, can yield in as much as 3 ng DNA (*12*).Furthermore, DNA has been shown to remain persist on surfaces for several days prior to collection, making touched items a valuable source of forensic information (*13*).

Given the very small number of nucleated cells in touched items, the cell type that contributes DNA to the STR profile is difficult to identify. While the fragmented and condensed nuclear DNA from skin cells may contribute to the profile, it is also likely that skin flakes serve as a vector for extracellular DNA

transfer from other body surfaces (nose, mouth, and eyes) as well as from sebaceous and sweat glands (*11, 14, 15*). Therefore, cells from other areas and bodily fluid may be present in so-called 'touch' samples, and may represent a contributing source of DNA (*11*).

Regardless of the cell type that is the source of the DNA detected, typically the DNA recovered from touched items originates from more than one person. This is likely because multiple individuals may handle these items such as knives, bats, guns, hammers, or window frames, for example. Since 2006, which marked the implementation of High Sensitivity DNA testing into routine casework at the OCME, thousands of samples with no visible biological staining have been processed for 'touch' DNA and the majority of these samples were found to be mixed. This manner of testing encompasses enhancements of every step from collection and to analysis of DNA (*3-6*). These involve a specialized swab to maximize DNA recovery (*4*), an extraction method that maximizes DNA recovery (*5*), and a highly sensitive method of quantitation (*16*). For violent crimes, this also includes increased cycles and replicates of PCR, optimized PCR and electrophoresis conditions, and specialized interpretation guidelines and quality control standards (*3, 17-19*). Using all of these high sensitivity methods in 2010, 1,990 DNA samples collected from touched items of evidence from violent crimes were amplified. Of these, 29.3% were either single source or mixtures where a foreign major donor could be determined. 17.6% were consistent with elimination samples being either single source, or resulting in a mixture where the deduced major donor was consistent with the victim. Although there was some foreign component present, it was too sparse to obtain a DNA profile. 38.2% were mixtures where no determination of individual contributor profiles could be made. The remaining 14.9% were inconclusive due to too few or too many alleles. Therefore, it is possible that the interpretation of roughly 60% of all samples amplified from touched items could be improved with a method of separating the cellular contribution of DNA contributors prior to DNA extraction and amplification.

A powerful tool to separate mixed cellular material prior to DNA extraction and amplification is micromanipulation. Micromanipulation refers to techniques that use microscopy and physical manipulation techniques, often in conjunction with specific staining, to remove cells of interest from an undesirable background. One widely described method of such separation is Laser Capture Microdissection (LCM) (*20*). LCM involves microscopic visualization of cell types followed by laser cutting of desired elements followed by capture, using physical force or the assistance of an adhesive polymer, into a collection vessel. Numerous platforms are available for these separations all which have unique methods for the specific cutting and capture. In our lab, we use the P.A.L.M. ®, Positioning and Ablation with Laser Microbeams (Carl Zeiss MicroImaging, Bernreid, Germany). This system uses an ultraviolet (UV) laser coupled to an inverted microscope. Samples can be visualized under bright field or fluorescent light, allowing for a wide range of staining techniques to be used. The UV laser, when brought into focus, provides a narrow beam which cuts and ablates surrounding undesired material. The P.A.L.M. ® uses the force of a quick pulse of diffuse UV light to catapult the sample into a collection vessel where the DNA or RNA of the separated material can be extracted. LCM has been successful in OCME's laboratory and in others for the separation of sperm and epithelial cell mixtures from mock sexual assault evidence, as well as mixed sex epithelial mixtures discerned by using chromosome-specific Fluorescent *In-Situ* Hybridization (FISH) staining (*21-25*). Due to size restrictions of the microscope platform, LCM separations are limited to material that can be applied to microscope slides. The P.A.L.M.® system is able to separate material applied to standard glass slides, specialized membrane coated slides, which provide a support backbone for the material of interest, and metal framed slides, which contain a polymer membrane without a glass support (*26*).

Manual microdissection techniques have also been widely used in biomedical research to examine mutations and gene-expression of pure cellular subpopulations (*27-31*). These techniques involve preparing a slide or whole

specimen and observing under microscopy followed by manual scraping or plucking of cellular material by manual means such as a sterile hypodermic needle (*32*). Manual microdissection techniques are less expensive than laser-assisted systems and may be adopted into the forensic biology lab more readily. It has recently been described in the forensic arena as an alternative to LCM (*33-36*). Microtweezers with or without adhesive microspheres have been used for microscopic separations with great success as well (*33, 36*). While LCM is generally considered to provide higher resolution and specificity, a manual method may be advantageous for materials or surfaces that can not be placed on a traditional microscope, such as cloth cuttings or objects such as handles. Furthermore, since no slide preparation is necessary the cells that should be separated can be visualized in their native flakes, possibly leading to better separation of material from individual contributors where homogenous cell morphology is present, as each flake may have been deposited by a different individual.

The robotically-assisted micromanipulator aureka® (Aura Optik, Jena, Germany) integrated to the Axiozoom® (Carl Zeiss AG, Göttingen, Germany) was custom fitted with a pair of microtweezers on a robotic arm. The motorized micromanipulator can move in four directions: left-right, forward-backward, up-down, and diagonally. This micromanipulator was compared to manual microdissection techniques.

Independent of the method of cell separation, using trace amounts of DNA for forensic testing can, by nature, involve very low amounts of DNA. During traditional analysis, quantitation is a necessary step to provide optimal STR typing results. Since the number of cells captured by micromanipulation techniques can be estimated and the quantity of DNA within a cell can be estimated, quantitation is likely redundant and represents a potential for DNA loss. One-tube extraction and amplification of samples removed by micromanipulation has the potential not only to streamline DNA workflows, but also prevent post-extraction DNA loss from manipulation. Direct PCR has been

used in microbiology for decades, by placing colonies of bacteria directly into the PCR reaction to identify species or gene mutations (*37*). Direct PCR has been shown to reduce the time-to-profile of swabs of clothing and reducing cost (*38*). Sperm and epithelial cells from mock-sexual assault evidence have been successfully profiled using this method (*39*). Furthermore profiles of low levels of deposited DNA on various substrates were improved over those that had undergone a separate extraction and quantitation step, presumably due to DNA loss in the latter scenario (*40*).

1.3 Statement of Hypothesis

So-called “touch-DNA” is typically obtained from items commonly handled or grabbed such as the handles of weapons, tools used to gain access to a safe, and clothing with no apparent biological staining. This evidence is recovered in the investigation of a wide variety of criminal offenses including homicide, sexual assault, felony assault, and property crime cases. As the evidence is often touched by multiple persons, perhaps multiple perpetrators and/or the victim(s), often the results include DNA contributions from several individuals.

Deconvolution of these mixtures particularly when dealing with initially small or trace amounts of cellular material, and thus DNA (100 pg or less), requires care and may be complex. Sometimes, the individual DNA profiles of multiple contributors cannot be determined, or may be consistent with the victim, and as a result may not be informative. For example, by using high sensitivity methods in 2010, 1,990 DNA samples collected from touched items of evidence from violent crimes were amplified. Of these, 29.3% were either single source or mixtures where a foreign major donor could be determined. 17.6% were consistent with elimination samples being either single source, or resulting in a mixture where the deduced major donor was consistent with the victim. Although there was some foreign component present, it was too sparse to obtain a DNA profile. 38.2% were mixtures where no determination of individual contributor profiles could be made. The remaining 14.9% were inconclusive due to too few or too

many alleles. Therefore, it is possible that the interpretation of roughly 60% of all samples amplified from touched items could be improved with a method of separating the cellular contribution of DNA contributors prior to DNA extraction and amplification.

Micromanipulation refers to techniques that use microscopy and physical manipulation techniques, often in conjunction with specific staining, to remove cells of interest from an undesirable background. Several microscopy and micromanipulation techniques are available to potentially separate individual contributors based on cell type or location on an item. Sperm and epithelial cells may be distinguished by morphology. Skin flakes may group by the placement of individual contributors on the object, while specialized staining techniques allow visual discrimination of male from female originating cells of the same morphology. These methods may allow the separation of microscopically visible mixture contributions before they inadvertently become pooled in a DNA extraction.

To this end it was proposed to provide situational and substrate-specific recommendations for the use of three micromanipulation techniques which have been described in the literature, and to develop a highly efficient method of direct PCR which will be universal to obtaining the Short Tandem Repeat (STR) profiles of the individual contributors to these substrates.

This evaluative study involved several phases: first, studies were conducted determining effective strategies for cell lifting from items that have been touched or contained scant amounts of cellular material using manual, laser and robotically assisted micromanipulations. During the second phase various DNA extraction and STR amplification techniques were compared, to find the DNA processing method that maximizes DNA recovery from fingerprints that was also used on minute amounts of other cell types. The third phase was characterized by intensive research on fingerprints for forensic identification and included a correlation of fingerprint depositions with their obtained STR profiles, analysis of fingerprints on various substrates, and a time course study. The final phase of

this study assessed the ability to reduce the incidence of irresolvable mixtures, by swabbing sectioned areas of touched items as well as by collecting single epithelial and sperm cells and processing them independently.

Micromanipulation in concert with sectioning samples and processing them independently, could lead for mixed fingerprints and/or cell mixtures to deducible profiles, revealing a clear major contributor that may be database eligible, and/or, in best cases, even to single source profiles.

2. Methods

2.1 Sample Collection

This research project was approved by the New York City Department of Health and Mental Hygiene Institutional Review Board that oversees research involving human subjects for the Office of Chief Medical Examiner (OCME) (IRB# 12-058). Before donating a biological sample, such as buccal cells, sperm and/or fingerprints, each volunteer read and signed a consent form. Each volunteer was assigned a numerical code to anonymize the sample.

Buccal cells: Cotton swabs were used to collect cells from the inside of the cheek. Cells were spread onto a PEN membrane slide. Immediately afterwards the cells were put on a water steam to spread them on the surface of the slide, followed by heat-fixation at 56^{o}C for 2 min and staining with NFR (Nuclear Fast Red) for 10 min. After washes with H_2O and EtOH the slide was placed on a Thermobrite® StatSpin® (Iris, Westwood, MA) for 56^{o}C for 2 min. Slides were stored at room temperature until use.

Sperm: Volunteers provided seminal fluids that were kept at 4^{o}C until use. Using a cotton swab, cells were spread onto a PEN membrane slide. Immediately afterwards the cells were fixed by heat at 56^{o}C for 2 min and stained with NFR (Nuclear Fast Red) for 10 min. After washes with H_2O and EtOH the slide was placed on a thermobrite for 56^{o}C for 2 min. Slides were stored at room temperature until use.

Fingerprints: Volunteers were asked to refrain from washing their hands for at least two hours prior sampling. Samples were taken by pressing right and left thumbs on various substrates [glass, plastic (sheet protector made of polypropylene), paper, metal (US Quarter dollar made of Cu and Ni), and tape] for few seconds.

After deposition of fingerprints, some were examined under an Olympus SZX-16® stereomicroscope (Olympus of the Americas, Central Valley, PA) to observe the individuals' propensity to shed skin cells and a score of 1 to 5 (sparse to dense shedding) was assigned.

(For detailed step-by-step protocols that were used for this research study see appendices.)

2.2 Spheres to Pick and Transfer Single Cells and Fingerprints

Microglobes and glue: protein-coated spheres SoloHill Hillex® (Solo Hill Engineering, Ann Arbor, MI) CT, SoloHill (collagen and ProNectin F coated), Cytodex™ 3, a collagen coated dextran microglobe (GE Healthcare, Upsala, Sweden), "Pritt" glue (Henkel AG&Co, Düsseldorf, Germany), "Elmer's stick" (Elmer's Products, Inc., Westerville, OH), Arabic gum (Winsor & Newton, London, UK) in glycerol 1:5 to 1:9, and rubber cement (Elmer's Products, Inc., Westerville, OH).

Tape: Latent print tape (Remco, Lancaster, CA), DIFF-lift tape (Lynn Peavey Forensics), fingerprint lifting tape (SIRCHIE, Youngsville, NC), "tesa" tape (Tesa, Hamburg, Germany), and Scotch tape (Scotch, St. Paul, MN).

Needles: Hypodermic needle to scrape cells and tungsten needle to scrape cells and to carry spheres.

(For detailed step-by-step protocols that were used for this research study see appendices.)

2.3 X/Y FISH

Pre-treatment of PEN and PET membrane slides: UV light irradiated slides were cleaned with 100% EtOH. 1 ml of 0.01% poly-L lysine (Sigma-Aldrich, St. Louis, MO) was spread over the membrane and left at room temperature for 1 h, followed by three rinses with water. Slides were dried for 2 h.

Cell preparation: Cuttings from male and female buccal swabs were placed in separate spin tubes, containing 500 µl 1x PBS+cellulose (Sigma-Aldrich, St. Louis, MO) and incubated in a thermomixer (Eppendorf International, NY, NY) at 900 rpm for 1 h at 37^{o}C. The swabs were transferred to spin baskets of which the membranes were removed and placed above the cell solution. The tubes were centrifuged at 10,000 rpm for 10 min. Then the spin baskets and supernatant were discarded. The cells were washed with 500 µl 1x PBS and resuspended in 10 µl Carnoy's fixative (Methanol and Acetic Acid 3:1). Male and female cells were mixed and spread over a 1 cm^2 marked area on the pre-treated membrane slide. The slide was steamed above a water bath for 5-10 sec, following heat fixation by placing the slide on the thermobrite (Iris, Westwood, MA) for 2 min at 56^{o}C. The slide was baked in a biological oven for 2.5 h at 60^{o}C, following incubation for 1 min in 70% EtOH, drying at room temperature. Slides were stored in desiccators.

X/Y FISH: Reusable perfusion chambers were attached to the marked region on the slide, filled with 500 µl of pepsin in a buffered solution and incubated for 1 h at 37^{o}C. Then the chamber was removed and the slide was washed in PBS/$MgCl_2$ solution [1 M $MgCl_2$ (50 ml) in 950 ml PBS] for 5 min, following incubation in 2.5% formalin and PBS for 5 min. The slide was immersed in 70%, 85%, and 100% EtOH, respectively, for 1 min each step, and dried on air. The DNA was denaturated by immersing the slide in denaturing formamid-solution for 5 min at 73^{o}C, followed by washes for 1min with 70%, 85%, and 100% EtOH. For the hybridization, the slide was warmed to 47^{o}C and 10 µl probe solution was added to the cells. A cover slip was applied to allow the solution to spread evenly. The hybridization occurred at 42^{o}C for 45 min and up to several hours.

The slide was washed in 0.4 x SSC at 73ºC for 2 min and in 2 x SSC/0.1% IGEPAL (NP-40) for 1 min. After air drying 10 µl DAPI was applied and the slide was for at least 30 min incubated at minus 20ºC to increase signal intensity.

2.4 Micromanipulation on Single Cells and Fingerprints

P.A.L.M. (Carl Zeiss, MicroImaging, Bernreid, Germany) Laser Capture Microdissection (LCM): magnification up to 400 fold was used for collection of single or groups of cells from PEN membrane-coated slides.

aureka® robotically-assisted micromanipulator (Aura Optik, Jena, Germany) integrated to the Axiozoom® (Carl Zeiss AG, Göttingen, Germany) (magnification up to 200 fold) custom fitted with a tool box on a robotic arm adapted to carry a pair of microtweezers, or a holder for a tungsten needle or microsurgical blade. The motorized micromanipulator can be moved in four directions: left-right, forward-backward, up-down, and diagonally. This instrument was used to pick up single cells, swab small areas (on slide or other object), cut (tape or membrane), and for transfer to reaction tubes.

Manual microdissection using the Axiozoom®: Manual scraping or lifting of cellular material using tools such as a sterile hypodermic needle, tungsten needle, or microtweezers coupled with adhesive microspheres. In addition, cells were manually cut out of sticky tape or membrane slides using a microsurgical blade (FEATHER Safety Razo Co, Ltd. Osaka, Japan) or the X-Acto knife (Dick Blick Art Material, Galesburg, IL).

Swabbing fingerprints: Fingerprints on glass or other substrates, such as plastic, metal, and paper, were swabbed under the Olympus SZX-16® stereomicroscope (magnification up to 40 fold) using a small portion of a cotton swab (Dynarex, Orangeburg, NY), and for tiny areas a sphere made of rubber cement. The prints were swabbed using water or 5% Triton X-100 (Sigma-Aldrich, USA). Fingerprints on lifting tape (SIRCHIE) were swabbed under the Axiozoom® stereomicroscope using 10 µl of prepGem® tissue extraction mixture diluted 1:2 in H_2O.

2.5 DNA Extraction and Quantification

One-tube extraction: Samples (swabs containing a single fingerprint) were incubated in 20 µl digestion buffer (3% trehalose, 1.44 mg/ml Proteinase K, and 1mM DTT) in a thermomixer (Eppendorf International, NY, NY) at 56ºC and 600 rpm for 45 min, followed by 10 min at 95ºC, and 5 min on ice.

High-sensitivity extraction: Swabs were incubated in a digestion buffer containing 0.05% SDS and 0.8 mg/ml Proteinase K. Following incubation on the Eppendorf Thermomixer® at 1400 rpm for 30 min at 56ºC, samples were then incubated without shaking for 10 min at 99ºC and 4ºC. Samples were concentrated and purified using a Microcon® 100 filter (Millipore, Billerca, MA) twice, which was pretreated with 200 µl of fish sperm DNA (Crescent Chemicals, Islandia, NY) of 5 ng/ µl. DNA was eluted with 20 µl UV irradiated water.

ZyGem extraction: 5% Triton X-100 was used to swab fingerprints. Swabs were incubated in 20 µl of prepGem® tissue extraction mixture (ZyGem, New Zealand) for 15 min at 75ºC, followed by 5 min at 95ºC using a GenAmp 9700 thermal cycler (Life Technologies, Applied Biosystems, Foster City, CA).

(For detailed step-by-step protocols that were used for this research study see appendices.)

Chelex DNA extraction from sperms: Sperm cells were incubated in extraction buffer, containing 200 µl of 5% Chelex (from a well-resuspended Chelex solution), 1 µl of 20 mg/ml Proteinase K, and 7 µl of 1 M DTT for 2 h at 56ºC, followed by vortexing at high speed for 10-30 s. Samples were then incubated for 8 min at 100ºC and vortexted for 10-30 s. After centrifugation for 2 min at 10-15,000 g, the samples were concentrated and purified using a Microcon® 100 filter (Millipore, Billerca, MA) twice, which was pretreated with 200 µl of fish sperm DNA (Crescent Chemicals, Islandia, NY) of 5 ng/ µl. DNA was eluted with 20 µl UV irradiated water.

(For detailed step-by-step protocols that were used for this research study see appendices)

DNA quantification: Following extraction, DNA was quantified using *Alu*-based real-time PCR for human DNA adapted from Nicklas and Buel (*16*). 2 µl extracted DNA was used as template in a 25 µl reaction using SYBR-Green (Life Technologies Molecular Probes, Grand Island, NY) and Ampli-*Taq* Gold (Life Technologies) on a Rotorgene™ Q (Qiagen, Valencia, CA). Quantification was performed in triplicate, duplicate or singletons.

2.6 Amplification of STR Loci

Samples were amplified using AmpF*l*STR® Identifiler® PCR Amplification Kit for 28 (ID28) or 31 (ID31) cycles or AmpF*l*STR® Identifiler® Plus PCR Amplification Kit (Life Technologies Applied Biosystems) for 32 (ID+) cycles. The PCR per reaction contained 2.5 µl Primer Mix, 5 µl Reaction Mix, 0.5 µl Ampli*Taq* Gold DNA Polymerase (5 U/µl), and 5 µl extracted DNA as template. For one-tube extracts, the $MgCl_2$ concentration was increased by 0.25 mM. A negative control was used for each amplification method, and if positive, the entire batch was disregarded. Samples were amplified either once or in triplicate. When amplified in triplicate, the alleles in at least two of three amplifications were considered part of the composite (consensus) profile. Only these alleles were assigned to the DNA profile for the sample. For samples that were amplified once, all labeled alleles were assigned to the STR profile (*3*).

2.7 Capillary Electrophoresis and Analysis

The amplified PCR products were separated on a 3130*xl* Genetic Analyzer (Life Technologies Applied Biosystems) at 3 kV for 20 s. Samples with overblown signals were reinjected at 1 kV for 22 s, and samples with low signals were reinjected at 6 kV for 30 s. Data analysis was performed using GeneMapper v. 4.0 software (Life Technologies Applied Biosystems). The peak amplitude threshold in GeneMapper was set to 75 RFUs detection. Peak ratio cut off value for tetra-nucleotide markers was set to 0.1.

2.8 Data Analysis

Data analysis was performed using SPSS version 21 (IBM, Armonk, NY) and Microsoft Excel.

3. Results

3.1 Collection of Single Cells

Cells from individual contributors can often be discriminated on microscopic level by cell morphology or location, and with the assistance of specific staining techniques. Effective strategies were determined to transfer single cells coupled with direct PCR using AmpF*l*STR® Identifilier® Plus (ID+) for STR typing and individualization. Microdissection was carried out by preparing a slide or whole specimen. The cells were stained using NFR, since the dye works in the visible light spectrum. DAPI (4',6-diamidino-2-phenylindole) staining was tested but not further used, since DAPI is excited with UV-light, which can affect the DNA (pyrimidine dimerization) and can lead to degradation, thereby affecting the downstream results (% STR profile). The stained cells were observed and selected under the microscope and cellular material was lifted by using tools such as a sterile hypodermic needle, or microtweezers coupled with adhesive microspheres. (see Appendix 1 for step-by-step protocol: Preparation of cells for micromanipulation)

3.1.1 Microcarriers to Collect Single Cells

Microcarriers are tiny spheres with a range of 90-300 µm in diameter. They are held by tweezers, whereby the correct position must be controlled with a mirror. The microcarriers can attach to a selected cell and hold it with greater force than the cell holds on to the surface. Thereby selected single cells can be collected and transferred into a reaction tube.

Three types of commercially available microglobes from Solo Hill Engineering were tested. These included polystyrene beads coated with collagen, ProNectin® F, and CT, a modified proprietary coating. As well as Cytodex™3 (GE

Healthcare) a dextran microglobe coated with collagen. In addition, office glue (Pritt, Henkel AG&Co, Düseldorf, Germany) and homemade spheres [rubber cement (Elmer's Products, Inc., Westerville, OH) and Arabic gum (Winsor & Newton, London, UK) mixed with glycerol (1:5 to 1:9)] were tested.

Adhesive properties of microglobes:

The microglobes from Solo Hill Engineering were easy to handle. However, it was difficult to lift cells consistently and the adhesive force was insufficient for the selected material to remain on the carrier long enough to be deposited into the reaction tube (Figure 1). Heating, drying, or cooling according to the manufacture's suggestion did not increase adhesiveness, including glue pre-treatment, using "Elmer's" glue (Elmer's Products, Inc., Westerville, OH) or "Pritt". Therefore, the microglobes from Solo Hill Engineering were not further considered for single cell picking.

The microglobes Cytodex™3 were soft and tend to burst under the pressure of the tweezers, which makes them difficult to handle. They were too small to pick more than one cell per globe.

Home-made spheres were generated of Arabic gum diluted in glycerol (1:5-1:9), rubber cement, "Pritt", and "Elmer's" glue. Arabic gum exhibited adhesive properties to lift single cells and came easily off the needle when transferring the cells into the reaction tube (Figure 2). Rubber cement was less adhesive and could not be used to pick up single buccal or sperm cells, but it was suitable for swabbing tiny areas to collect skin cells (partial fingerprints) (Figure 3). "Pritt" glue was very sticky and lifted single cells efficiently. However, when used to swab cellular material of fingerprints it smeared and left stains on the glass slide. In addition, it could not be easily released into the reaction tube because of its stickiness. "Elmer's" glue, which is soluble in water, was difficult to roll to a sphere, and could not be used to lift cells.

Compatibility of microglobes with PCR reaction

The microglobes from Solo Hill Engineering, and Cytodex™3, Arabic gum, rubber cement, and the glues "Pritt" and "Elmer's" were tested with AmpF*l*STR®

Identifiler® Plus (ID+) for PCR inhibition. Cytodex™3 showed PCR inhibition if more than one microglobe was added to the reaction tube.

Outcome for further studies:

Single epithelial (buccal) and sperm cells can be collected with a sphere of Arabic gum, which is held by a tungsten needle. The sphere can be easily released in a reaction tube. Arabic gum does not interfere with downstream PCR reactions. Fingerprints and dried skin flakes can be swabbed using a sphere of rubber cement, for partial prints, or cotton threads for the whole print. The sphere and thread is held by tweezers. Rubber cement showed no PCR inhibition. Fingerprints on lager objects, too large to put under the microcope, can be transferred by tape. (see Appendix 2 for step-by-step protocol: Collection of single cells - micromanipulation: 2.1 Arabic gum to lift single epithelial and sperm cells, 2.2 Rubber cement for swabbing partial fingerprints, and 2.3 Cotton spheres to swab entire fingerprint)

3.1.2 Needles to Collect Single Cells

Hypodermic and tungsten needles can be used to scrape cells. Skin cells and flakes do not exhibit electrostatic interactions and are therefore difficult to transfer by using a needle. Upon amplification using AmpF*l*STR® Identifiler® Plus (ID+), directly or after DNA one-tube extraction, no data or sporadic results were obtained. Epithelial (buccal) cells spread onto glass slides can be scraped off using the sharp part of the hypodermic needle. Tungsten needles are not as effective as hypodermic needles due to their shape. Amplification, following the one-tube extraction, using ID+ led to full STR profiles. However, the use of needles to collect single cells is not recommended, since the scraping procedure is difficult to adjust and takes too many cells.

3.1.3 Tape to Collect Fingerprints and Skin Cells

Tapes can be used to preserve positions of cells collected from an object for further scanning and for digitalizing fingerprints. Fingerprints on large objects,

such as door knobs, or parts of a wall, can be lifted by tape and transferred to be analyzed by microscope (stereomicroscope or Axiozoom®). Fingerprints can then be swabbed to extract DNA and generate STR profiles. Five types of tape were tested for the collection of fingerprints and skin cells: Latent print tape (Remco), DIFF-lift tape (Lynn Peavey Forensics), fingerprint lifting tape (SIRCHIE), “tesa”, and Scotch tape.

Latent print tape and DIFF-lift tape were either thick or textured and difficult to use for manual cutting and therefore excluded from further studies. Fingerprint lifting tape (SIRCHIE), “tesa”, and Scotch tape could be used for manual cutting (Figure 4). However, previous studies evaluating different tapes for fiber uptake, saturation, recovery, ease of cutting the tape and lifting of the patch showed that Scotch did not perform well, and therefore was also excluded. Tapes can only be used for manual and robotically-assisted micromanipulation, not for laser capture microdissection (LCM), since the laser cannot cut through the tape.

Compatibility of tapes with PCR reaction

The two tapes, fingerprint lifting tape from SIRCHIE and “teasa” were tested in AmpF*l*STR® Identifiler® Plus (ID+) STR Amplification and showed no PCR inhibition. Since the fingerprint lifting tape from SIRCHIE is used for forensic applications, we selected this for further tests.

STR profiles from fingerprints lifted with tape

Prints can be swabbed off tapes (SIRCHIE) under the stereomicroscope by using cotton threads, held by tweezers, and 10 µl of a 1:2 ZyGem dilution in water, following DNA extraction using the ZyGem protocol and STR amplification by ID31. The comparison of fraction profiles of fingerprints deposited directly on tape (n=37) and fingerprints deposited on glass slides that were lifted by tape (n=41) showed that both methods led to database eligible profiles (≥0.7). As shown in Figure 5, direct deposition on the tape resulted in more complete profiles; however the difference was not significant (t-test, p=0.345). (see Appendix 2 for step-by-step protocol: Collection of single cells - micromanipulation: 2.4 Tape to lift fingerprints off larger objects)

3.1.4 X/Y FISH

Mixed samples of male and female contributors from sexual assault cases most often consist of spermatozoa and epithelial cells where morphological differences enable easy separation at cellular level. Sometimes, materials on items of evidence take the form of mixed epithelial cells, such as in azoospermic sexual assaults or assaults involving licking, spitting or biting where separation based on size and shape is impossible. However, male and female cells can be separated by sex using X/Y Fluorescent *in-situ* hybridization (FISH), which stains the X and Y chromosomes differently, leaving the cells intact. In X/Y FISH, the fluorescently probes pass through the cellular membranes and migrate into the nucleus where they bind to their target sequence. A fluorescence microscope is used to visualize the bound probes. Female cells are identified by two orange signals while male cells by an orange and green signal.

In this study modifications to Abbott's X/Y FISH protocol were examined for compatibility with PEN and PET membrane slides in order to use the PALM. However, the membrane of the PEN slides showed auto-fluorescence. As a conclusion PET membrane slides were used. The slides were UV-irradiated and treated with poly-L lysine in order to keep the cells on the membrane throughout the staining procedure. The cells were treated with pepsin for one hour. The hybridization of the fluorescent X/Y probes was extended overnight enabling efficient penetration and hybridization.

Figure 6 taken from the PALM shows that epithelial cells can be separated by X/Y FISH. In combination with microscopy and micromanipulation FISH techniques can be used for cell separation of various contributors.

3.2 Protocol for DNA Extraction and STR Amplification from Fingerprints

Fingerprints and touched items are important sources of DNA for STR profiling. However, there are some fundamental difficulties in working with these samples, including their substantial variability in quality and quantity.

3.2.1 DNA Extraction

Fingerprints are considered as challenging forensic samples since they often contain low amounts of template DNA and can vary greatly in their DNA quantity and quality. Different extraction protocols were compared in order to find a reliable method with a high recovery rate. This method could then also be applied on other cellular material, where only a few cell will be sampled.

Fingerprints deposited on glass slides were swabbed in their entirety with a moistened cotton swab, using either water or 5% Triton X-100, under the Olympus stereomicroscope SZX-16. DNA was isolated using one of the three protocols: (i) HighSens, (ii) one-tube, and (iii) ZyGem. The HighSens protocol is routinely used at the OCME to extract DNA from touched samples and in-house studies have shown that this procedure is superior to other routinely used methods (*5*). HighSens extraction includes multiple steps, it is time consuming, it requires a transfer of the sample into new tubes, and it includes a purification step. Purification and tube exchange make a sample more prone to contamination and could lead to some loss of the DNA. Therefore, direct methods (one-tube and ZyGem) were also tested. Using direct methods, a sample is extracted in one step, whereby only one tube is used and the sample is not further treated (e.g. purified). The one-tube protocol was also developed in-house. Briefly, the sample is incubated for 45 min at 56^{o}C in 3% trehalose, ProtK and DTT (see Methods: 2.5 DNA extraction and quantification). The ZyGem DNA extraction kit is commercially available. The extraction is short and simple. ZyGem utilizes a modified enzyme with optimal activity at high temperature (75°C). After 15 min incubation the DNA solution can be used for PCR (DNA quantification and/or STR amplification).

DNA extracted from single fingerprints was quantified using the *Alu*-based real-time PCR, adapted from Nicklas and Buel (*16*). This method has a 30% error rate. Samples were quantified in single or triple measurements, of which the median was used. Single measurements led to higher values, most likely due to

outliers (Figure 7). However, the difference was significant (Fisher exact test p=0.028).

Using Triton X-100 to swab the print did not improve the recovery of the one-tube extraction method (Figure 7).

The highest DNA recovery was obtained by using the HighSens DNA extraction procedure, followed by Zygem and the one-tube method (Figure 7). Using Fisher exact test, HighSens extraction was significantly better than one-tube ($p=6.5x10^{-5}$) and Zygem (p=0.001).

3.2.2 STR Amplification and Partial Profiles

Following DNA extraction, the DNA of the fingerprint samples were amplified using ID28, ID31, and ID+. ID28 is routinely used at the OCME to amplify samples with DNA concentrations >20 pg/µl, while ID31 is used as a part of the High Sensitivity DNA testing protocol. ID+ is, as per suggestion of the manufacturer, more resistant to PCR inhibitors (*41*). ID28, ID31 and ID+ were compared in order to find the amplification protocol that would reliably result in database eligible profiles (≥70%) and also to be used on other minute cellular material.

The samples extracted using the one-tube extraction protocol were amplified with ID28, ID31 and ID+, while samples extracted using HighSens and ZyGem protocols were amplified with ID31 and ID+. Of 756 fingerprint samples processed, 710 revealed to be single source and 46 were mixtures. Most of the STR profiles obtained were partial. Profiles that were at least to 70% complete were considered as valuable, since such profiles could be used to search databases. For samples of which less than 2 pg/µl of DNA was obtained (n=275), only 21 resulted in profiles that were database eligible. Therefore, samples with higher or equal concentration to 2 pg/µl DNA (n=436) were compared to find out which of the STR amplification protocols would lead to the best outcome.

Using the one-tube extraction procedure followed by ID31 amplification, almost 70% of tested samples (≥2 pg/µl) reached at least 70% completeness. Using ID+

increased the outcome to approximately 90% (Figure 8), while using ID28 showed a decrease. For ZyGem extracted samples ID+ had no effect. ID31 resulted in over 90% of samples that were at least to 70% complete (Figure 8). The same trend was observed for samples extracted with HighSens, but was lower than for the ZyGem extraction (Figure 8).

The one-tube extraction followed by ID31 was considered as baseline for the comparison. Using logistic regression one-tube extraction followed by ID+ increased the outcome significantly ($p=7.23x10^{-6}$), while ID28 decreased it, but not significantly (p=0.43). When ZyGem extracted samples were amplified with ID31 and ID+, the outcome increased in comparison to the one-tube extraction followed by ID31, but not significantly (p=0.285). HigSens extracted samples followed by any of tested amplifications showed a significantly decrease in the outcome (p=0.005). ZyGem DNA extraction followed by ID31 led to the best outcome.

Including the results of the extraction method, showed that the best protocol is Zygem DNA extraction followed by ID31, while HighSens followed by ID31 and the one-tube extraction followed by ID+ have a similar but lower success rate. [see Appendix 3 for step-by-step protocol: DNA extraction: 3.1 DNA extraction from swabs of fingerprints and epithelial cells (ZyGem)]

3.3 Single Fingerprint Analysis

Touched or grabbed items, such as weapons, knife, or other objects without obvious staining can be used as evidence in a variety of criminal cases. Therefore, fingerprints can be considered as a potential source of DNA for forensic identification.

3.3.1 Partial Fingerprints

In order to deconvolute mixtures of two or more people from touched items and to generate DNA profiles of individual contributors, individual cell clumps were collected. Five skin clumps/flakes were collected from a single fingerprint, DNA

was extracted and STRs amplified. The obtained DNA profile was partial, but very low, barely reaching 20% completeness. The collection of five skin flakes was repeated five times, resulting in similar outcomes (Figure 9). Partial profiles, below 70% were considered as not database eligible.

The collected skin cells could have gone through full keratinization and nucleus fragmentation, as part of apoptosis and therefore hardly any DNA would be found to generate DNA profiles from (*7*). However, amplifiable DNA on touched items could be extracellular from sweat or sebaceous glands (*15*). Consequently, a small area of a fingerprint was swabbed. A fingerprint was divided in eight similar size sections, which were separately processed for DNA extraction and STR amplification (Figure 9). The qualities of the resulting STR profiles showed a broader variation, some of them reached 40% completeness (Figure 9). Based on this data, it was concluded that fingerprints should be collected in their entirety, by swabbing, in order to obtain database eligible profiles.

3.3.2 Visible Deposition and Fraction Profile

Fingerprints on touched items can greatly vary in their density for deposited skin cells. To determine whether a shedding level, sparse or dense, could be used as a predictor of successful DNA amplification, DNA profiles were obtained and compared to the prior assigned shedding levels.

Prior to donating, volunteers were asked to refrain from washing their hands for at least 2 h. Fingerprints of left and right thumbs were deposited on separate glass slides, visually examined under the stereomicroscope Olympus SZX-16, prior to swabbing and DNA extraction. Based on the density of skin cells within the fingerprint immersion the quality scores 1-5 (sparse to dense shedding) were assigned to each fingerprint. Occasionally, the scores 1 and 5 were assigned. Most fingerprints were scored 3 or 4. Nevertheless, some individuals seem to be better shedder than others (Figure 10). Over 110 fingerprints were analyzed per person. Following visual examination, fingerprints were swabbed, DNA was extracted using ZyGem, and the samples were quantified and amplified using

ID31. Great variation in profile completeness was obtained for each volunteer. However, the outcome revealed that some volunteers that were considered as better shedders, such as volunteer 10, showed STR profiles of lower quality range. Other volunteers, considered as not so good shedders, as volunteer 14, led to STR profiles that were of better quality (Figure 10). Using linear regression, increase in the amount of DNA of 2.24 pg/µl ($p=0.20$) and in the percent of DNA profile of 4.2% ($p=4.8x10^{-4}$) per one unit in shedding score was observed, but because of the great variability in profile qualities from different shedding scores recovered, the shedding score was not considered to be used as a reliable predictor for profiling success (*42*).

3.3.3 Analysis of Right versus Left Fingerprints

Skin cells together with extracellular DNA, sweat and oil are usually deposited by touching an object. The deposition was swabbed, DNA recovered and STR profiles of various qualities obtained. Left and right hands were analyzed separately to test whether a right handed person will tend to deposit more material by holding an object with the right hand, or less because of the frequently use of this hand over the left.

Over 500 fingerprints from left and right thumbs were collected and shedding scores of 1 to 5 were assigned. The fingerprints from four volunteers were tested. Despite the fact that most fingerprints were assigned a score of 2, 3 or 4, the score varied between volunteers (Figure 10). A significant difference between shedding score of donors' right and left hand could not be found for any of the volunteers (Chi- square test showed p- values of >0.4 for all volunteers) (*42*).

In order to find out if there is also no difference for the resulting fraction STR profiles, additional >600 fingerprints from three volunteers were collected. The three volunteers were right handed. The prints of the left and right thumbs were swabbed separately, extracted by using ZyGem and amplified with ID31. The STR profiles obtained from their left hands were slightly more complete that from their right hands (Figure 11). However, t-tests showed that the difference

between left and right hand for volunteer 1 was not significant (p=0.206), but for other two (p=0.001 for volunteer 2 and p=0.020 for volunteer 3, see Figure 11). T-tests for all fingerprints showed that the difference of profiles obtained from left and right thumbs was significant (p<0.001).

In conclusion, while the shedding score is not a reliable predictor for profiling success and cannot be used to determine DNA recovery, the study showed that by using one hand more frequently, cells and extracellular DNA may be shed on various locations and could lead to slightly less complete profiles.

3.3.4 Fingerprints on Various Substrates

When a large number of touched items from crime scenes are submitted for analysis, time and resources are best spent targeting those items with the highest likelihood of success of obtaining database eligible DNA profiles. To test the effect that various substrates may have on cellular material, fingerprints were placed on glass, metal (a cleaned US Quarter dollar, made of Cu and Ni), paper, and plastic (a sheet protector, made of polypropylene), items that are commonly touched in households and offices.

Prior to fingerprint collection, the substrates were, except for paper, decontaminated using 10% bleach, followed by water, and 70% EtOH. In addition, all substrates were UV irradiated for 30 minutes. Volunteers were asked to refrain from washing their hands 2 h prior to sample collection. The fingerprints were swabbed three days after their deposition on the tested substrates; DNA was extracted using ZyGem followed by ID31 amplification. Approximately 30 prints per substrate were collected and analyzed. The completeness of DNA profiles ranged widely. The best profiles were obtained from glass, followed by plastic and paper, and no profiles were obtained from metal (Figure 12).

The outcome suggests that metal ions may cause DNA degradation (*43*). When prioritizing touched item evidences for examination glass would be the preferred substrate, since it showed the highest fraction profiles and database eligible DNA profiles compared to the other types of substrates tested.

3.3.5 Time Course

Touched evidences from the crime scenes are delivered to the laboratory for examination in packaged condition. Several days can pass prior to evidence collection and its laboratory examination. Despite that, evidence can be exposed to various conditions at the crime scene prior to packaging. Time and environment may have an effect on the qualities and quantities of DNA recovered from such evidence. A time course study was performed to find out if the fingerprints over time provoke quality changes in STR-profiles. In addition, it was evaluated, whether in-house conditions, fingerprints on glass slides stored in open boxes which are exposed to dust, could affect the quality of the resulting STR data.

Volunteers were asked to refrain from washing their hands for at least 2 h prior to sampling. Glass slides with the prints were stored in clean boxes for defined time periods: 1, 3, 10, 20, and 40 days. The boxes were either left open to expose the prints to in-house conditions such as dust or closed to protect the prints. The DNA was isolated from these prints using ZyGEM and amplified using ID31.

Figure 13 shows that the fraction of profile varied greatly. However, over time fingerprints result in smaller fractions of database eligible profiles (≥70% complete). ANOVA analysis showed significance between these time points and t-tests specified that the decrease of fraction profile was not significant between day 1 and day 3, but the decrease was significant between day 1 and day 10, as well as for longer time periods.

Prints that were exposed 10 days to in-house conditions led to lower fraction profiles than the fingerprints that were protected (t-test: $p=0.001$). After 20 days, a difference between open and closed boxes could not be observed (Figure 14). In order to get meaningful STR profiles, touched items should be processed as soon as possible.

3.3.6 Three Person Mixtures

Routinely used methods for sampling evidence from violent crimes could be touched by multiple persons, often generating mixtures of which no individual profiles of the contributors can be determined. Deconvolutions may be possible if a major donor is obvious. However, individual DNA profiles of more than one contributor are most difficult to determine and results may not be informative.

It is most likely that the interpretation of samples amplified from touched items can be improved by sectioning the evidence and handling them separately (*44*). This may increase the probability of obtaining two persons mixtures with a major contributor that may be deductible or even single source profiles from the same evidence, leading to DNA profiles of more than one, or ideally to all contributors of a mixture.

For the three people fingerprint mixture study, an empty beer (amber glass) bottle was cleaned using 10% bleach, water, and 70% EtOH. The body of the bottle was etched into six equivalent sections and touched consecutively by three volunteers. Before touching, the volunteers were asked to refrain from washing their hands for at least 2 h prior to sampling. The order of volunteers touching the bottle was recorded and changed. The bottle was examined under an Olympus SZX-16® stereomicroscope. Fingerprints were swabbed from each etched section and processed using ZyGem extraction, followed by ID31. Each swabbed section was treated as an individual sample, amplified in triplicate and analyzed following the high sensitivity interpretation protocol for single source and mixed samples (*3*). Briefly, the composite profile contained alleles that occurred in at least two out of the three amplifications and was used to classify a sample as a single source or a mixture of two or three donors. Samples with three or more repeating alleles per locus were interpreted as mixtures. Thirty-six bottles were tested. Six samples were generated per bottle and the six composite profiles were used to determine the number of people that touched the bottle (Figure 15).

Our analysis showed that most samples of each bottle identified at least a two person mixtures. Over 50% of the tested bottles showed involvement of at least 3

persons (Table 1). Further it was found that the last person who touched the bottle did not mainly contribute to the profiles obtained. The sampling method, six samples per bottle, led to some single source and deducible profiles with at least 70% completeness (Table 1). Importantly, in some cases (14%) two different profiles (≥70% complete) were obtained from the same bottle (Table 1).

3.4 Comparison of Micromanipulation Instruments: PALM, Axiozoom, and Aureka®

The PALM (Positioning and Ablation with Laser Microbeams, from Zeiss), an inverted microscope that can be used with a magnification up to 400x, allows the isolation of individual cells (Figure 16). It features laser capture microdissection (LCM), visualization under the microscope, and uses laser energy to transfer areas or cells of interest. Typically, cells are selected under the microscope, cut and catapult into the lid of a reaction tube. The Axiozoom (Zeiss) is a high-resolution stereomicroscope (magnification up to 200x), to which the aureka® (Aura Optik), a motorized micromanipulator, could be connected (Figure 16). The Axiozoom is equipped with a UV-light unit that contains several filters allowing adjustment for different surface materials and morphological assessment of nuclei. Further, it memorizes positions of stage, light and magnification until deleted (*33*). The robotic arm aureka® can be moved in four directions: x-axis (left-right), y-axis (forward-backward), z-axis (up-down), and m-axis (diagonally). The attached tweezers can be opened and closed. The aureka® also memorizes positions for tweezers and tungsten needle until turned off, and then a resetting is needed (*36*). However, throughout work, it may lose its precision of the x,y,z directions and should be turned off in order to reset the instrument. The Axiozoom stage was designed by us and includes a platform that holds two microscope slides, one is to make the spheres from Arabic gum/glycerol and the other contains the cells or cell mixtures. This platform can be rotated. The stage also contains a rack for reaction tubes allowing the direct transfer of the cells (Figure 17).

In order to compare PALM, Aziozoom handled manually or with the robotic arm, aureka®, STR profiles were generated from 10 buccal cells (n=25 repeats). Figure 18 shows that the fraction STR profiles using the PALM were low, while the Axiozoom operated manually or with the aureka® had values between 0.7 and 0.8, which were considered database eligible. These differences were significant (t-test). This outcome was corroborated by testing sperm cells. Ten and twenty sperm cells were selected, DNA extracted and STR profiles generated using ID31 (at least 9 times repeated, as shown in Figure 19). Ten sperm cells led to better fraction profiles than ten epithelial (buccal) cells. Most fraction profiles were higher than 70%. Sperm cells are smaller than epithelial cells but they are compact and the DNA is protected by a cell wall that could make the collection easier. Twenty sperm cells led to fraction profiles higher than 70%. Again, the results using the PALM were not as good as using the Axiozoom, operated manually or with the aureka® (Figure 19). These differences were significant (t-test).

Taken together (Table 2), the PALM can be used for single cell picking (laser cutting and transfer into reaction tube) of epithelial (buccal) cells and sperm cells. The Axiozoom operated manually or with aureka®, the robotic arm, can be used to swab fingerprints, either directly or off the tape, to cut tapes that contain prints or cells, and for single cell [epithelial (buccal) and sperm cells] lifting by using spheres. Manual swabbing of larger areas is preferred than using aureka®, since it is faster.

4. Conclusion

4.1 Discussion of Findings

Fingerprints can be collected from a wide variety of evidences of crime scenes, such as handles of weapons, tools used in criminal cases, and clothing with no apparent staining. They could be of tremendous value in forensic biology. Their pattern, when deposited on objects is used for identification. Fingerprints could also be a source of DNA (*4*). However, many touched items processed through

routine workflow showed great variation in quality and quantity of the extracted DNA (*2*). Therefore, they are potentially unreliable for forensic DNA testing. Optimized DNA extraction and STR amplification protocols are needed, in order to obtain quality profiles that can be used for CODIS from the aforementioned types of evidence.

Entire fingerprints were used to compare direct and multi-step DNA extraction protocols, followed by STR amplification using AmpF*l*STR® Identifiler®. It was found that DNA extraction using ZyGem (prepGem® tissue extraction kit), a direct DNA extraction procedure that features a modified enzyme, which works at high temperatures followed by AmpF*l*STR® Identifiler® PCR using 31 cycles led to the highest fraction profiles (*42*). (see appendices for specific step-by-step protocols)

Items received from crime scenes for examinations can be a combination of two or more people that handled an object or a blend of body fluids resulting in DNA mixtures. If similar amounts of donors' DNA are present, the resulting STR profiles cannot be deduced to their individual donors. However, single cell picking could be a method to separate a cell and therefore eliminate the appearance of mixed samples, by generating single source profiles. Most likely, one cell would result in partial STR profiles that cannot be used to search databases, such as CODIS (*45*). This research utilized the AmpF*l*STR® Identifiler® PCR Amplification Kit that amplifies 15 autosomal loci plus Amelogenin, for a maximum of 30 autosomal alleles per donor. Profiles that were at least 70% complete were considered valuable, since such profiles could be used to search databases.

Micromanipulation can be used for separation of mixed cellular material prior to DNA extraction and STR amplification. Commercially available microcarriers were tested for single cell collection (*33, 36*). However, the microspheres from SoloHill (Solo Hill Engineering, Ann Arbor, MI) were not adhesive to lift cells and the microspheres from Cytodex™3 (GE Healthcare, Upsala, Sweden) showed PCR inhibition. Water soluble glue may be used to transfer cellular material from touched items (*46*). Testing various glues showed that small spheres made from

Arabic gum mixed with glycerol were appropriate for single cell collection and rubber cement was suitable for swabbing small areas. Both materials showed no PCR inhibition.

Five skin flakes barely reached 20% completeness of STR profiles. Swabbing sections of fingerprints resulted in a similar outcome, but showed greater variation, which may come from the contribution of extracellular DNA (*15*). Increasing the number to fifty skin flakes can lead to full STR profiles (*46*). But most likely, some of these consist of keratin and may be non-nucleated (*10, 47*). This is supported by the outcome of this study: the shedding score, describing the deposition of a fingerprint from sparse to dense, is not a reliable predictor for profiling success (*42*). Therefore, it was concluded that fingerprints should be swabbed in their entirety. (see appendices for step-by-step protocols)

Further analysis showed that for right-handed persons fingerprints of the left hand, the non-dominant hand, led to slightly higher fraction profiles, not supporting the results of another study (*14*). Volunteers for this study were asked to refrain 2 h from washing their hands prior to donating, which may lead to higher deposition of the non-dominant hand, while the dominant hand may encounter some abrasion. The outcome was not significant for each person tested.

This study investigated the impact of various substrates on DNA recovery and the resulting STR profiles upon touching. Previous studies documented successful DNA recovery from paper (*48*), as well as from wood, glass and metal. Glass showed the best results (*49*). Other studies couldn’t confirm this outcome and reported that the highest DNA yield was received from wood, followed by fabric and glass (*50*). Here, four substrates were tested: glass, paper, plastic and metal, a US Quarter dollar, showing that glass led to the best STR profile qualities, followed by plastic and paper. No profile was obtained from metal, assuming that metal ions (from Cu and Ni) may have an effect on DNA degradation (*43*).

It was also found that time and environment has an impact on the qualities and quantities of resulting STR profiles from fingerprints, leading to the conclusion that touched samples should be processed as soon as possible.

Each extraction and amplification batch tested contained a negative control and if positive the samples of this batch would have been discarded. Approximately 6% mixtures were detected and disregarded. We assume, since the negative controls were negative, one of the reasons for occurrence of these mixtures could be secondary DNA transfer. This rate is low compared to other studies, where the secondary transfer ranged from 10% to 85% (*50-55*), but could be a consequence of the protocol we utilized. The amplification was performed in triplicate and a consensus profile (alleles must be present in at least two of three amplifications) was used to assign alleles. This procedure eliminates spurious alleles of the costs of increased allele drop out (*56*).

In order to address the problem of DNA mixtures from touched items sectioning and independent processing of the several samples was suggested (*11, 44*). This approach was tested on a beer bottle, which has been sectioned into six parts and was subsequently touched by three persons. The outcome showed some single source and deducible profiles that were at least to 70% complete. This method could also be used for cellular mixtures, of sperm cells, sperm and epithelial cells, or epithelial cells that occur in some criminal cases. Micromanipulation could be used to separate cells by shape or, for similar cell types, in conjunction with staining methods. In this study, PALM and Axiozoom operated manually or with the robotic arm aureka® were compared (*33, 36, 57*). It was found that 10 buccal cells and 10 sperm cells were sufficient to generate database eligible STR profiles using the Axiozoom, a lower number than recently published (*57*). The Axiozoom is more flexible in its application and can also be used for tapes. If fingerprints are found on objects that are too large to observe directly under the microscope, tapes can be used to transfer the prints. Tapes have been widely used as sampling method in forensics (*58-60*).

In summary, the demanding problems of minute amounts of cellular material and mixtures of two or more persons in forensic applications were addressed in this study by research on single cell collections and fingerprints, investigations on DNA extraction and STR amplification, as well as on micromanipulation.

4.2 Implications for Further Research

Generating STR profiles from fingerprints is challenging due to the fact that very little cellular material is left with the print. Therefore, this study analyzed an enormous number of fingerprints. Still it would be interesting if other laboratories could confirm the outcomes that were generated by this study. This could help to advance to a more standardized protocol for handling fingerprints in forensic casework.

Validating the Axiozoom for forensic casework could be beneficial, since this instrument can be used on a wide variety of criminal cases, including property crime, and sexual assault cases, where mixed samples of two or more contributors may be deduced.

Further research testing more FISH probes for separating cellular mixtures may facilitate single cell collection of different contributors.

4.3 Implications for Policy and Practice

Interpretation of admixed STR profiles from multiple donors present a challenge for forensic laboratories. In particular, when dealing with initially small or trace amounts of cellular items, such as touched objects, which require care and may be complex. This study approached this challenge, by finding a method to collect single cells and the cellular material of fingerprints using instruments appropriate for micromanipulation, by separating samples and processing them independently, as well as by the examination of protocols to extract DNA and amplify STR loci to generate database eligible profiles.

5. References

1. Schneider, H., Sommerer, T., Rand, S., and Wiegand, P. (2011) Hot flakes in cold cases, *Int. J. Legal Med. 125*, 543-548.
2. van Oorschot, R. A., Ballantyne, K. N., and Mitchell, R. J. (2010) Forensic trace DNA: a review, *Investig. Genet. 1*, 14.
3. Caragine, T., Mikulasovich, R., Tamariz, J., Bajda, E., Sebestyen, J., Baum, H., and Prinz, M. (2009) Validation of testing and interpretation protocols for low template DNA samples using AmpFlSTR Identifiler, *Croat. Med. J. 50*, 250-267.
4. Prinz, M., Schiffner, L. A., Sebestyen, J. A., Bajda, E., Tamariz, J., Shaler, R. C., Baum, H., and Caragine, T. (2006) Maximization of STR DNA typing success for touched objects, *Int. Congr. Ser. 1288*, 651-653.
5. Schiffner, L. A., Bajda, E. J., Prinz, M., Sebestyen, J., Shaler, R., and Caragine, T. A. (2005) Optimization of a simple, automatable extraction method to recover sufficient DNA from low copy number DNA samples for generation of short tandem repeat profiles, *Croat Med J 46*, 578-586.
6. Budimlija, Z. M., and Caragine, T. A. (2012) Interpretation guidelines for multilocus STR forensic profiles from low template DNA samples, *Methods Mol Biol 830*, 199-211.
7. Gandarillas, A., Goldsmith, L. A., Gschmeissner, S., Leigh, I. M., and Watt, F. M. (1999) Evidence that apoptosis and terminal differentiation of epidermal keratinocytes are distinct processes, *Exp. Dermatol. 8*, 71-79.
8. Lowe, A., Murray, C., Whitaker, J., Tully, G., and Gill, P. (2002) The propensity of individuals to deposit DNA and secondary transfer of low level DNA from individuals to inert surfaces, *Forensic Sci. Int. 129*, 25-34.
9. Toothman, M. H., Kester, K. M., Champagne, J., Cruz, T. D., Street, W. S. t., and Brown, B. L. (2008) Characterization of human DNA in environmental samples, *Forensic Sci. Int. 178*, 7-15.
10. Kita, T., Yamaguchi, H., Yokoyama, M., Tanaka, T., and Tanaka, N. (2008) Morphological study of fragmented DNA on touched objects, *Forensic Sci Int Genet 3*, 32-36.
11. Wickenheiser, R. A. (2002) Trace DNA: a review, discussion of theory, and application of the transfer of trace quantities of DNA through skin contact, *J Forensic Sci 47*, 442-450.
12. Alessandrini, F., Cecati, M., Pesaresi, M., Turchi, C., Carle, F., and Tagliabracci, A. (2003) Fingerprints as evidence for a genetic profile: morphological study on fingerprints and analysis of exogenous and individual factors affecting DNA typing, *J. Forensic Sci. 48*, 586-592.
13. Raymond, J. J., van Oorschot, R. A., Gunn, P. R., Walsh, S. J., and Roux, C. (2009) Trace evidence characteristics of DNA: A preliminary investigation of the persistence of DNA at crime scenes, *Forensic Sci Int Genet 4*, 26-33.

14. Phipps, M., and Petricevic, S. (2007) The tendency of individuals to transfer DNA to handled items, *Forensic Sci. Int. 168*, 162-168.
15. Quinones, I., and Daniel, B. (2012) Cell free DNA as a component of forensic evidence recovered from touched surfaces, *Forensic Sci. Int. Genet. 6*, 26-30.
16. Nicklas, J. A., and Buel, E. (2003) Development of an Alu-based, real-time PCR method for quantitation of human DNA in forensic samples, *J. Forensic Sci. 48*, 936-944.
17. Gill, P. (2001) Application of low copy number DNA profiling, *Croat. Med. J. 42*, 229-232.
18. Gill, P., Whitaker, J., Flaxman, C., Brown, N., and Buckleton, J. (2000) An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA, *Forensic Sci. Int. 112*, 17-40.
19. Kloosterman, A. D., and Kersbergen, P. (2003) Efficacy and limits of genotyping low copy number (LCN) DNA samples by multiplex PCR of STR loci, *J Soc Biol 197*, 351-359.
20. Espina, V., Wulfkuhle, J. D., Calvert, V. S., VanMeter, A., Zhou, W., Coukos, G., Geho, D. H., Petricoin, E. F., 3rd, and Liotta, L. A. (2006) Laser-capture microdissection, *Nat Protoc 1*, 586-603.
21. Anslinger, K., Bayer, B., Mack, B., and Eisenmenger, W. (2007) Sex-specific fluorescent labelling of cells for laser microdissection and DNA profiling, *Int J Legal Med 121*, 54-56.
22. Axler-DiPerte, G. O., S. Singh, A. Caragine, T Prinz, M. Budimlija, Z. M. . (2011) Comparison and optimization of DNA recovery from sperm vs. epithelial cells using laser capture microdissection technology and an immunofluorescent staining system, *Forensic Science International: Genetics Supplement Series 3*, 224-225.
23. Di Martino, D., Giuffre, G., Staiti, N., Simone, A., Le Donne, M., and Saravo, L. (2004) Single sperm cell isolation by laser microdissection, *Forensic Sci Int 146 Suppl*, S151-153.
24. Elliott, K., Hill, D. S., Lambert, C., Burroughes, T. R., and Gill, P. (2003) Use of laser microdissection greatly improves the recovery of DNA from sperm on microscope slides, *Forensic Sci Int 137*, 28-36.
25. Seidl, S., Burgemeister, R., Hausmann, R., Betz, P., and Lederer, T. (2005) Contact-free isolation of sperm and epithelial cells by laser microdissection and pressure catapulting, *Forensic Sci Med Pathol 1*, 153-157.
26. Micke, P., Ostman, A., Lundeberg, J., and Ponten, F. (2005) Laser-assisted cell microdissection using the PALM system, *Methods Mol Biol 293*, 151-166.
27. Chang, W. C., Keller, C. G., and Sretavan, D. W. (2006) Isolation of neuronal substructures and precise neural microdissection using a nanocutting device, *J Neurosci Methods 152*, 83-90.

28. Choi, B. B., Goldstein, M., Moomjy, M., Palermo, G., Rosenwaks, Z., and Schlegel, P. N. (2005) Births using sperm retrieved via immediate microdissection of a solitary testis with cancer, *Fertil Steril 84*, 1508.
29. Huang, Q., Sacks, P. G., Mo, J., McCormick, S. A., Iacob, C. E., Guo, L., Schaefer, S., and Schantz, S. P. (2005) A simple method for fixation and microdissection of frozen fresh tissue sections for molecular cytogenetic analysis of cancers, *Biotech Histochem 80*, 147-156.
30. Moskaluk, C. A. (2001) Microdissection of histologic sections : manual and laser capture microdissection techniques, *Methods Mol Med 50*, 1-13.
31. Wang, H. T., Ma, F. L., Ma, X. B., Han, R. F., Zhang, Y. B., and Chang, J. W. (2006) Differential gene expression profiling in aggressive bladder transitional cell carcinoma compared to the adjacent microscopically normal urothelium by microdissection-SMART cDNA PCR-SSH, *Cancer Biol Ther 5*, 104-110.
32. Kristiansen, G. (2010) Manual microdissection, *Methods Mol Biol 576*, 31-38.
33. Bruck, S., Evers, H., Heidorn, F., Muller, U., Kilper, R., and Verhoff, M. A. (2011) Single cells for forensic DNA analysis--from evidence material to test tube, *J Forensic Sci 56*, 176-180.
34. Li, C. X., Wang, G. Q., Li, W. S., Huang, J. P., Ji, A. Q., and Hu, L. (2011) New cell separation technique for the isolation and analysis of cells from biological mixtures in forensic caseworks, *Croat Med J 52*, 293-298.
35. Pereira, J., Neves, R., Forat, S., Huckenbeck, W., and Olek, K. (2012) MtDNA typing of single-sperm cells isolated by micromanipulation, *Forensic Sci Int Genet 6*, 228-235.
36. Schneider, C., Muller, U., Kilper, R., and Siebertz, B. (2012) Low copy number DNA profiling from isolated sperm using the aureka(R)-micromanipulation system, *Forensic Sci Int Genet 6*, 461-465.
37. Tjhie, J. H., van Kuppeveld, F. J., Roosendaal, R., Melchers, W. J., Gordijn, R., MacLaren, D. M., Walboomers, J. M., Meijer, C. J., and van den Brule, A. J. (1994) Direct PCR enables detection of Mycoplasma pneumoniae in patients with respiratory tract infections, *J Clin Microbiol 32*, 11-16.
38. Linacre, A., Pekarek, V., Swaran, Y. C., and Tobe, S. S. (2010) Generation of DNA profiles from fabrics without DNA extraction, *Forensic Sci Int Genet 4*, 137-141.
39. Meredith, M., Bright, J. A., Cockerton, S., and Vintiner, S. (2012) Development of a one-tube extraction and amplification method for DNA analysis of sperm and epithelial cells recovered from forensic samples by laser microdissection, *Forensic Sci Int Genet 6*, 91-96.
40. Swaran, Y. C., and Welch, L. (2012) A comparison between direct PCR and extraction to generate DNA profiles from samples retrieved from various substrates, *Forensic Sci Int Genet 6*, 407-412.
41. Romanini, C. F. M. R. C., M. L. Vullo, C. (2011) A comparison of AmpFlSTR Identifiler™ Kit versus AmpFlSTR Identifiler Plus™ Kit in

challenging bone samples by using normal and increased PCR cycle number, *Forensic Science International: Genetics Supplement Series 3*, 514-515.

42. Ostojic, L., Klempner, S. A., Patel, R. A., Mitchell, A. A., Axler-DiPerte, G. L., and Wurmbach, E. (2014) Qualitative and quantitative assessment of single fingerprints in forensic DNA analysis, *Electrophoresis 35*, 3165-3172.
43. Henle, E. S., and Linn, S. (1997) Formation, prevention, and repair of DNA damage by iron/hydrogen peroxide, *J Biol Chem 272*, 19095-19098.
44. Ballantyne, J., Hanson, E. K., and Perlin, M. W. (2013) DNA mixture genotyping by probabilistic computer interpretation of binomially-sampled laser captured cell populations: combining quantitative data for greater identification information, *Sci Justice 53*, 103-114.
45. Butler, J. M. (2006) Genetics and genomics of core short tandem repeat loci used in human identity testing, *J Forensic Sci 51*, 253-265.
46. Hanson, E. K., and Ballantyne, J. (2013) "Getting blood from a stone": ultrasensitive forensic DNA profiling of microscopic bio-particles recovered from "touch DNA" evidence, *Methods Mol Biol 1039*, 3-17.
47. Darmon, M. B., M. Lj. (1993) *Molecular biology of the skin: the keratinocytes*, San Diego : Academic Press, ©1993.
48. Sewell, J., Quinones, I., Ames, C., Multaney, B., Curtis, S., Seeboruth, H., Moore, S., and Daniel, B. (2008) Recovery of DNA and fingerprints from touched documents, *Forensic Sci Int Genet 2*, 281-285.
49. Pesaresi, M. B., L. Alessandrini, F. Cecati, M. Tagliabracci A. (2003) Qualitative and quantitative analysis of DNA recovered from fingerprints, *International Congress Series 1239*, 947– 951.
50. Daly, D. J., Murphy, C., and McDermott, S. D. (2012) The transfer of touch DNA from hands to glass, fabric and wood, *Forensic Sci Int Genet 6*, 41-46.
51. Cale, C. M., Earll, M. E., Latham, K. E., and Bush, G. L. (2015) Could Secondary DNA Transfer Falsely Place Someone at the Scene of a Crime?, *J Forensic Sci*.
52. Djuric, M., Valjen, T., Stanojevic, A., and Stojkovic, O. (2008) DNA typing from handled items, *Forensic Sci Int genet Supplement Series 1*, 411-412.
53. Helmus, J., Bajanowski, T., and Poetsch, M. (2016) DNA transfer-a never ending story. A study on scenarios involving a second person as carrier, *Int J Legal Med 130*, 121-125.
54. van Oorschot, R. A., Glavich, G., and Mitchell, R. J. (2014) Persistence of DNA deposited by the original user on objects after subsequent use by a second person, *Forensic Sci Int Genet 8*, 219-225.
55. Zoppis, S., Muciaccia, B., D'Alessio, A., Ziparo, E., Vecchiotti, C., and Filippini, A. (2014) DNA fingerprinting secondary transfer from different skin areas: Morphological and genetic studies, *Forensic Sci Int Genet 11*, 137-143.

56. Grisedale, K. S., and van Daal, A. (2012) Comparison of STR profiling from low template DNA extracts with and without the consensus profiling method, *Investig Genet 3*, 14.
57. Vandewoestyne, M., and Deforce, D. (2010) Laser capture microdissection in forensic research: a review, *Int J Legal Med 124*, 513-521.
58. Barash, M., Reshef, A., and Brauner, P. (2010) The use of adhesive tape for recovery of DNA from crime scene items, *J Forensic Sci 55*, 1058-1064.
59. Verdon, T. J., Mitchell, R. J., and van Oorschot, R. A. (2014) Evaluation of tapelifting as a collection method for touch DNA, *Forensic Sci Int Genet 8*, 179-186.
60. Zech, W. D., Malik, N., and Thali, M. (2012) Applicability of DNA analysis on adhesive tape in forensic casework, *J Forensic Sci 57*, 1036-1041.
61. Afonso Rabelo-Gonçalves EM, R. B., Robilotta Zeitune JM. (2014) Helicobacter pylori and Liver – Detection of Bacteria in Liver Tissue from Patients with Hepatocellular Carcinoma Using Laser Capture Microdissection Technique (LCM), In *Trends in Helicobacter pylori Infection* (Roesler, B. M., Ed.), InTech.

6. Dissemination of Research Findings

This work resulted in one publication and two additional publications are planned, and was presented on three meetings (oral presentations) as well as on several in-house educational seminars for approximate 150 criminalists at the OCME.

Publication:

Ostojic L, Klempner SA, Patel RA, Mitchell AA, Axler-DiPerte GL, Wurmbach E. (2014) Qualitative and quantitative assessment of single fingerprints in forensic DNA analysis. *Electrophoresis* Nov;35(21-22):3165-72.

Publications planned:

- Analysis of fingerprint samples, testing various conditions, for forensic DNA identification
- Micromanipulation techniques for cell mixture separation

Conferences:

- NEAFS conference 2014: Oral presentation: “Can ‘Direct’ PCR Method Improve DNA Analysis of Fingerprints?”, by Lana Ostojic, New York City Office of Chief Medical Examiner
- AAFS conference 2015: Oral presentation: “Assessment of Fingerprints for Forensic STR Analysis”, by Lana Ostojic, New York City Office of Chief Medical Examiner
- Green Mountain DNA Conference 2015: Oral presentation: “Comparison of PALM, Axiozoom and Aureka for single cell and fingerprint micromanipulation in forensic identification”, by Elisa Wurmbach, New York City Office of Chief Medical Examiner

7. Tables and Figures

7.1 Tables

Table 1: Outcome of three-person mixture

Sequence of touching the bottle	6 bottles: 10-12-14	6 bottles: 14-12-10	6 bottle: 14-10-12	6 bottles: 12-14-10	6 bottles: 10-14-12	6 bottles: 12-10-14
2p mixture (separate)	6 out of 6 bottles	6 out of 6 bottles	6 out of 6 bottles	6 out of 6 bottles	6 out of 6 bottles	6 out of 6 bottles
3p mixture (separate)	1 out of 6 bottles	2 out of 6 bottles	2 out of 6 bottles	3 out of 6 bottles	2 out of 6 bottles	4 out of 6 bottles
3p mixture (composite)	4 out of 6 bottles	3 out of 6 bottles	3 out of 6 bottles	6 out of 6 bottles	4 out of 6 bottles	5 out of 6 bottles
Single source profile (≥70%)	3 profiles of 2 bottles	7 profiles of 5 bottles	4 profiles of 3 bottles	2 profiles of 1 bottle	0	4 profiles of 3 bottles
Deducible profiles (≥70%)	9 profiles of 5 bottles	6 profiles of 4 bottles	5 profiles of 2 bottles	5 profiles of 4 bottles	7 profiles of 3 bottles	2 profiles of 2 bottles
Identified individuals per bottle (≥70%)	0 12 10/14 12 14 14	14 10 14 10/12 14 10	10 12 0 0 10 14	0 10/12 10/12 10 14 14	14 0 0 14 0 14	14 0 10 14 0 12/14

2 identified individuals per bottle: 5/36 (14%)
1 identified individual per bottle: 22/36 (61%)
0 identified individual per bottle: 9/36 (25%)

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Table 2: Comparison of Micromanipulation Instruments

Instrument	Cell type	Collection	
PALM	Epithelial cells (buccal) Sperm cells	Picking (laser cutting)	Single cells
Axiozoom/Manual	Fingerprints (direct/tape) Epithelial cells (buccal) Sperm cells	Swabbing Cutting tape Picking	Large/small areas Single cells
Axiozoom/Aureka	Fingerprints (direct/tape) Epithelial cells (buccal) Sperm cells	Swabbing Cutting tape Picking	(Large) small area Single cells

7.2 Figures

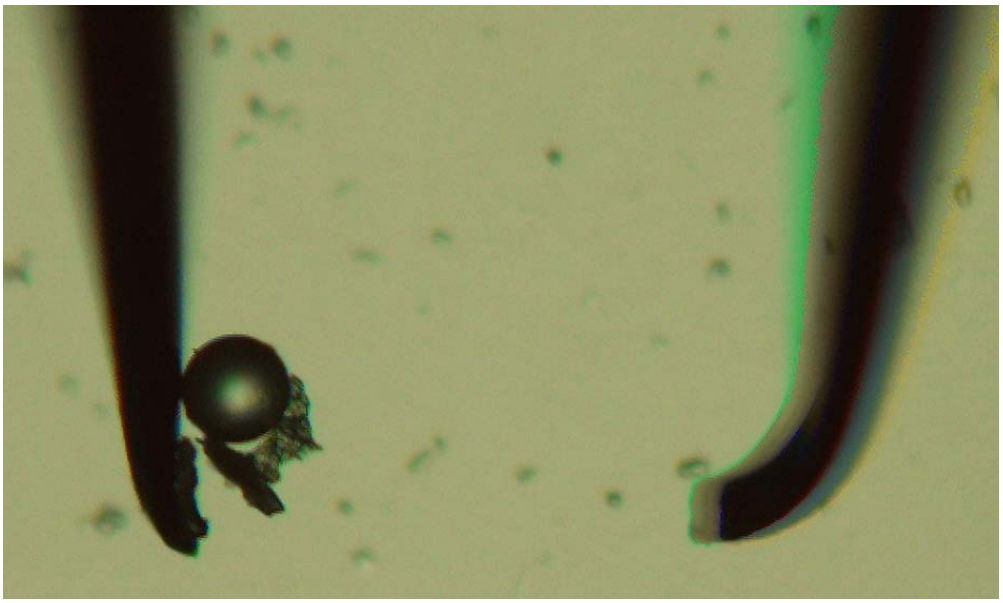

Figure 1: Skin cell flakes attached to a collagen coated polystyrene microglobe (Solo Hill Engineering) held manually with Dumont tweezers (115x magnification, Axiozoom).

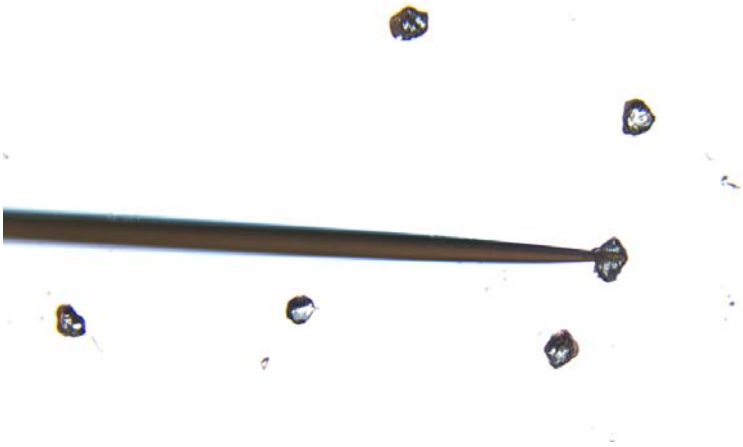

Figure 2: Arabic gum/glycerol spheres (approximately 200 µm in diameter, 80x magnification, Axiozoom) can be used to lift single cells. One sphere is held with the tip of the tungsten needle (Axiozoom).

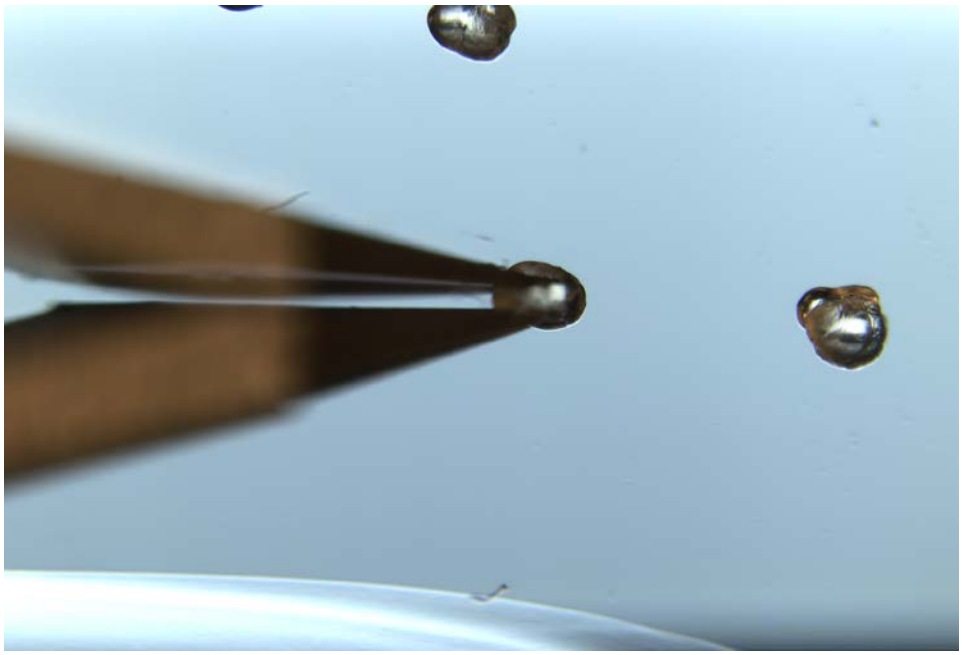

Figure 3: Aureka tweezers holding a rubber cement sphere. Spheres of rubber cement are suitable for swabbing tiny areas on glass slides, partial fingerprints (90x magnification, Axiozoom).

Figure 4: Fingerprint on SIRCHIE tape: Skin flake is visible (right panel), the tape was cut using an X-Acto knife (right panel) and removed (left) using Dumont tweezers (observed under Axiozoom, 20x magnification).

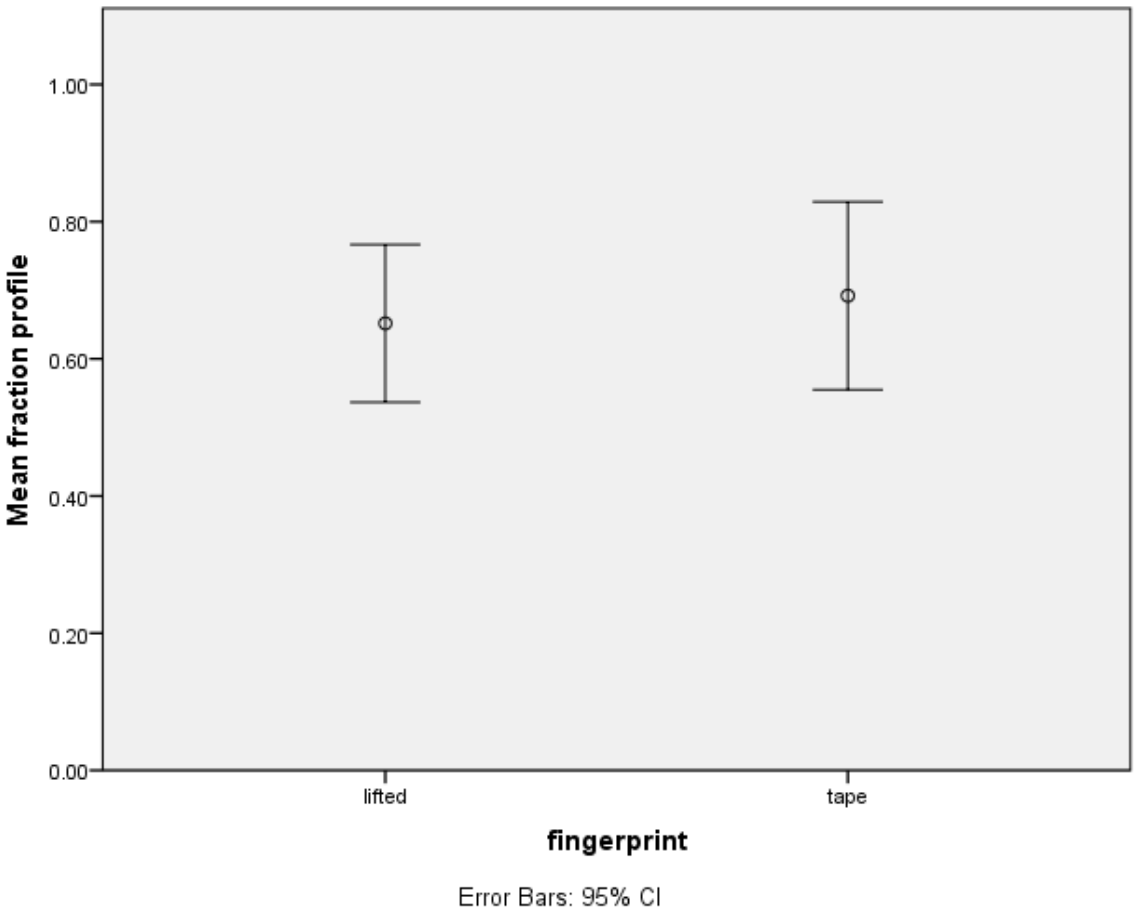

Figure 5: Distribution of fraction STR profiles obtained from swabbed fingerprints: lifted from glass slides using tape (lifted, n=41) and deposited directly on the tape (tape, n=37). T-test showed no significance (p=0.345). Analysis performed using SPSS.

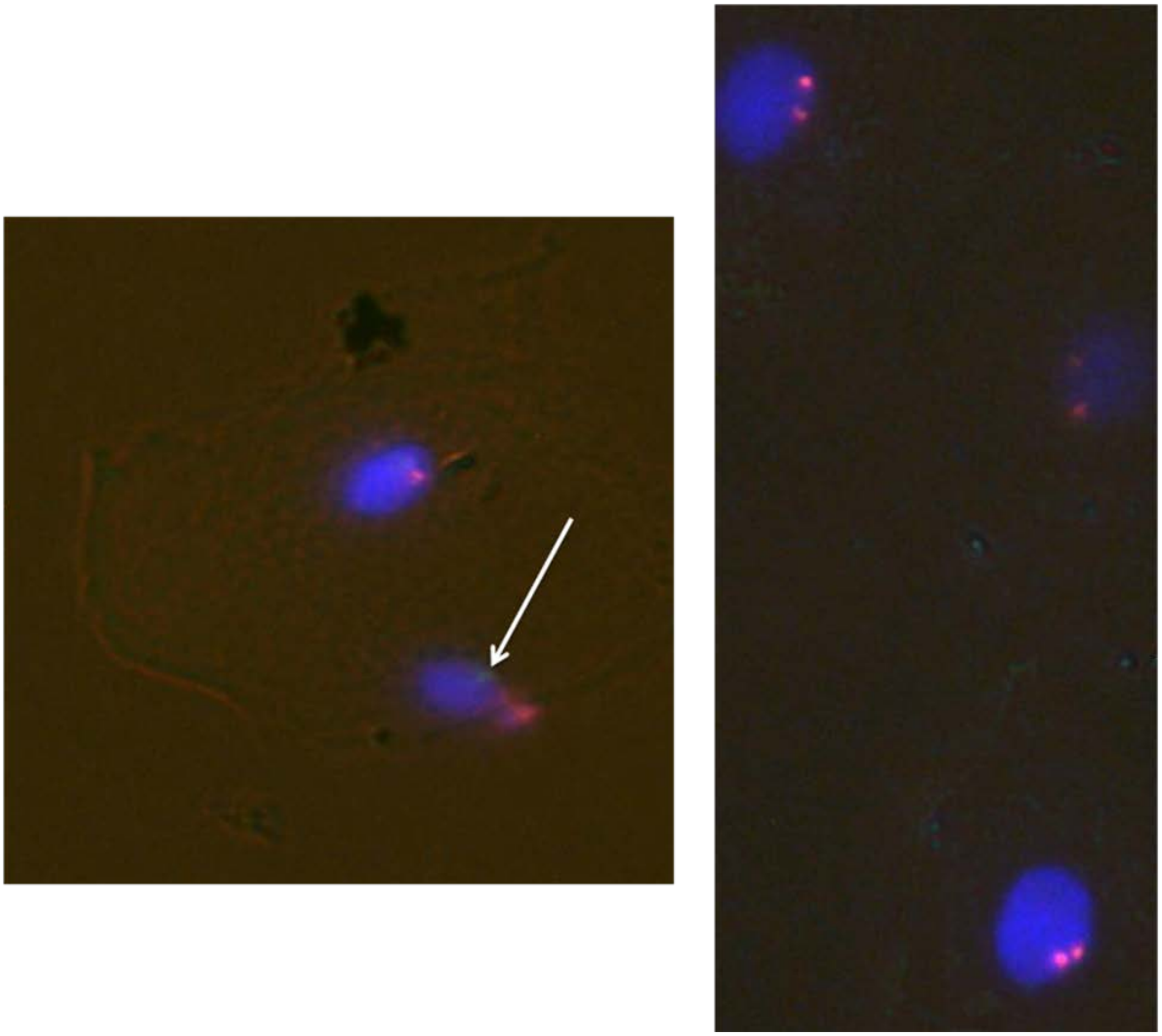

Figure 6: The cells are DAPI stained (blue, indicating the nucleus) showing orange signals for X- and green signals for Y-chromosome. The left panel shows male cells and the arrow points to a green signal. The right panel shows female cells, indicated by the two orange signals at each nucleus. (PALM, 40X, merge of laser, DAPI, FITC, and 43 HE filters).

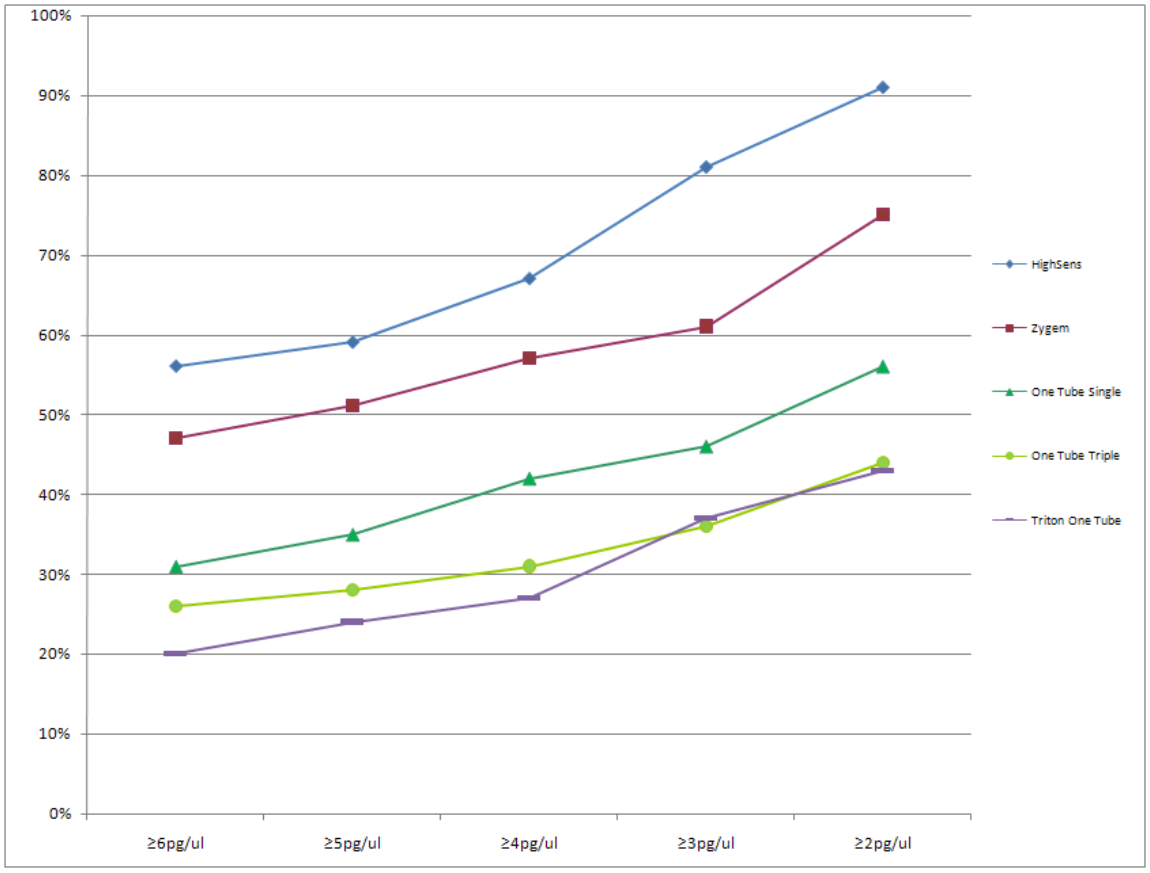

Figure 7: Distribution of measured DNA concentrations of fingerprint samples, swabbed with water or Triton X-100, extracted using one-tube, High-Sens, or Zygem methods, and quantified by using *Alu*-based real-time PCR (in singletons or triplicates).

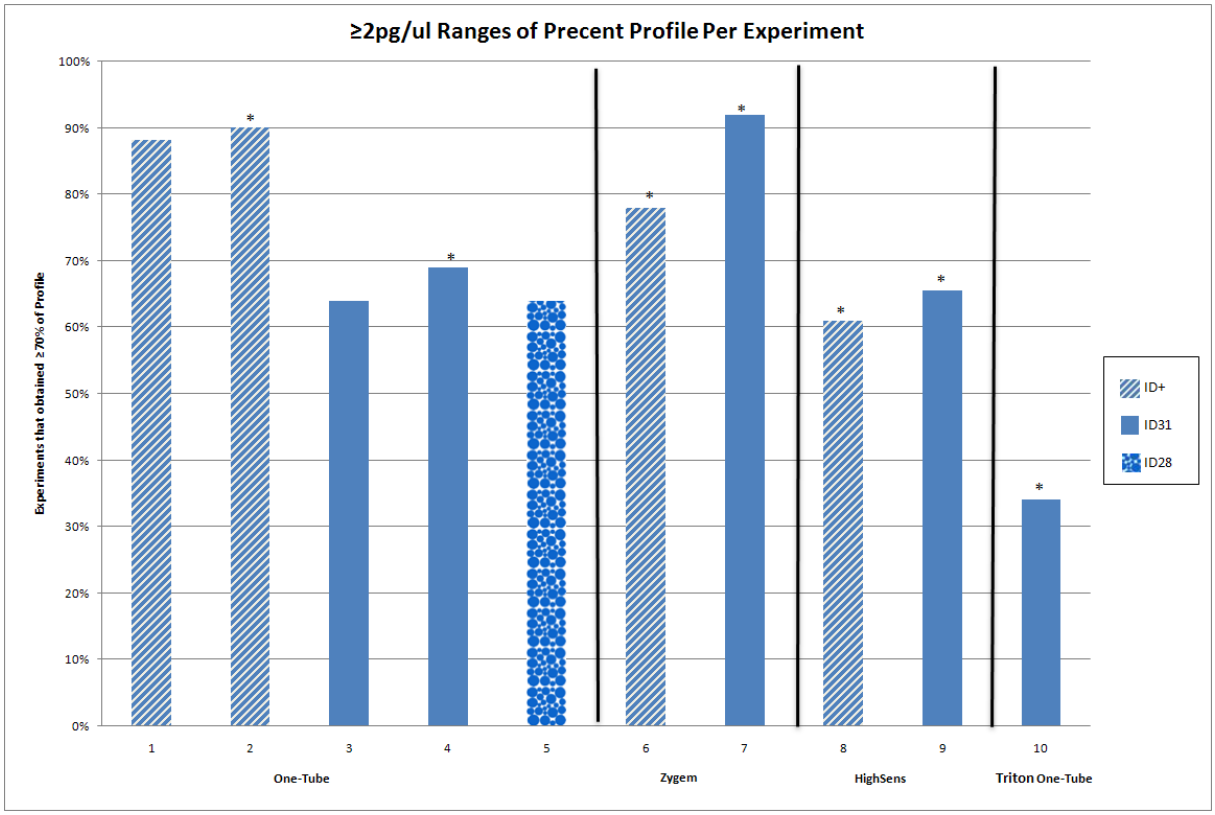

Figure 8: Percent of non-mixture fingerprint samples with DNA concentrations ≥2 pg/µl for which at least 70% of the donor's profile was obtained. * STR amplification was performed in triplicate.

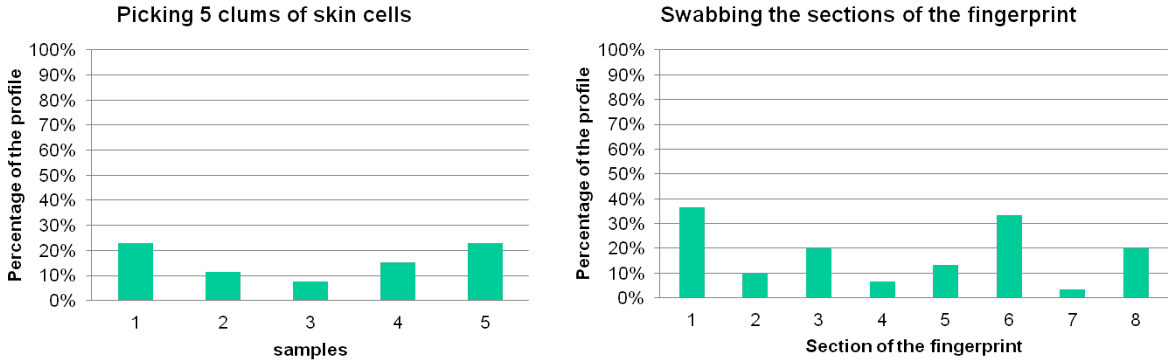

Figure 9: Left panel: Percent of DNA profiles obtained from 5 skin cell clumps/flakes tested in 5 independent experiments.
Right panel: Percent of DNA profiles obtained from parts of one fingerprint, which was divided in 8 similar size sections, which were processed independently.

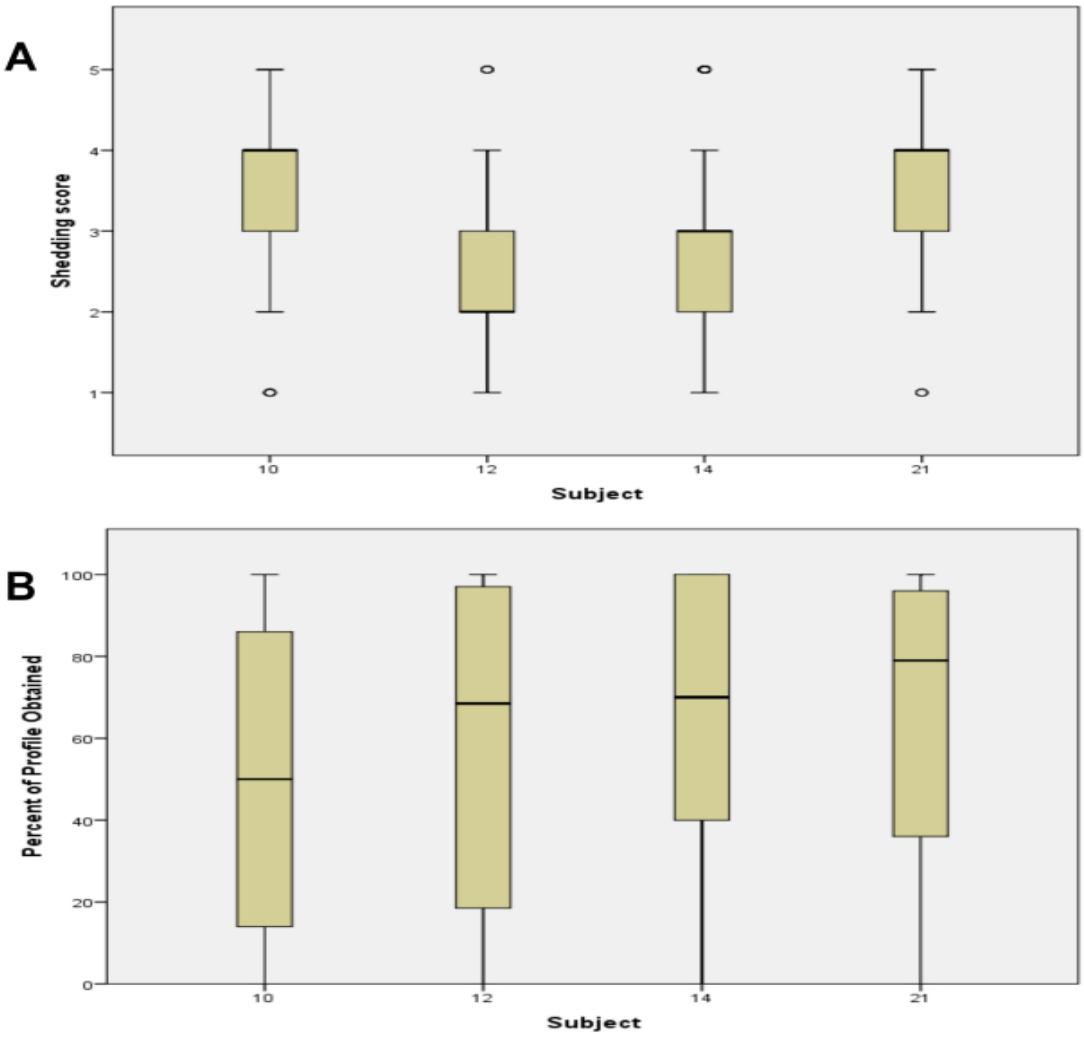

Figure 10: Single fingerprint analysis: (A) Distribution of shedding scores for fingerprints and (B) distribution of percent profile obtained for fingerprints collected from volunteers 10 (n=146), 12 (n=146), 14 (n=112), and 21 (n=120). Shown are median (dark line), 25th and 75th percentile (bottom and top of box), minimum and maximum (T-bars), and outliers (circles). Analysis performed using SPSS.

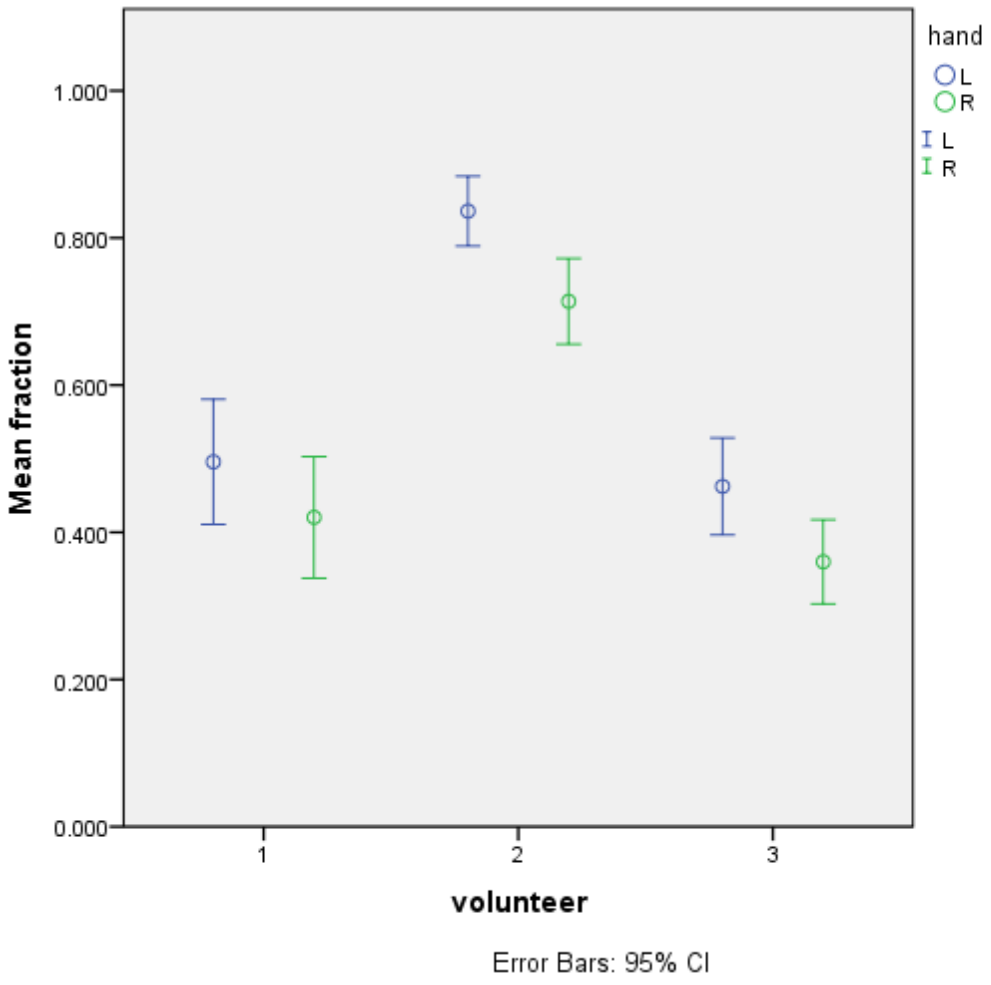

Figure 11: Fraction profiles from left (blue) and right (green) thumbs from three right handed volunteers are shown. Their left fingerprints resulted in slightly higher fraction profiles.

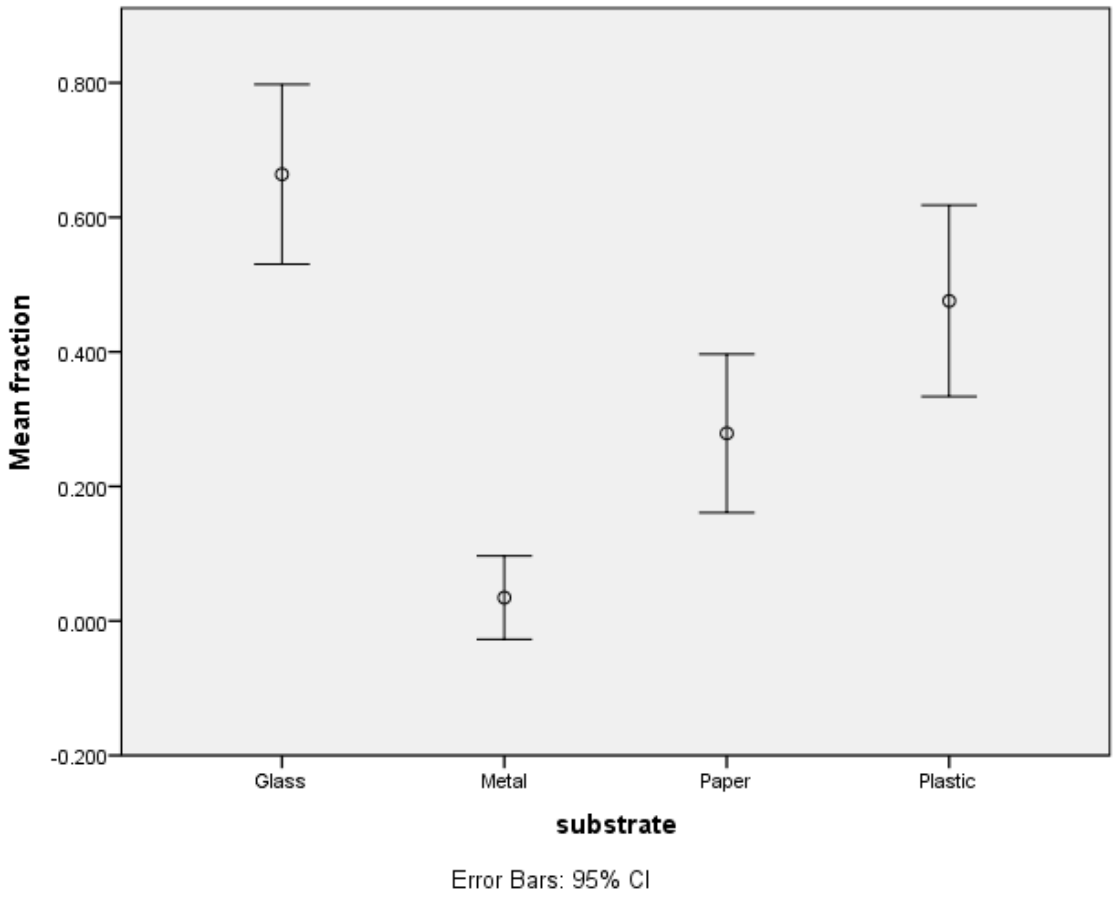

Figure 12: Fraction profiles from fingerprints on glass (n=32), metal (n=32), paper (n=32), and plastic (n=27).

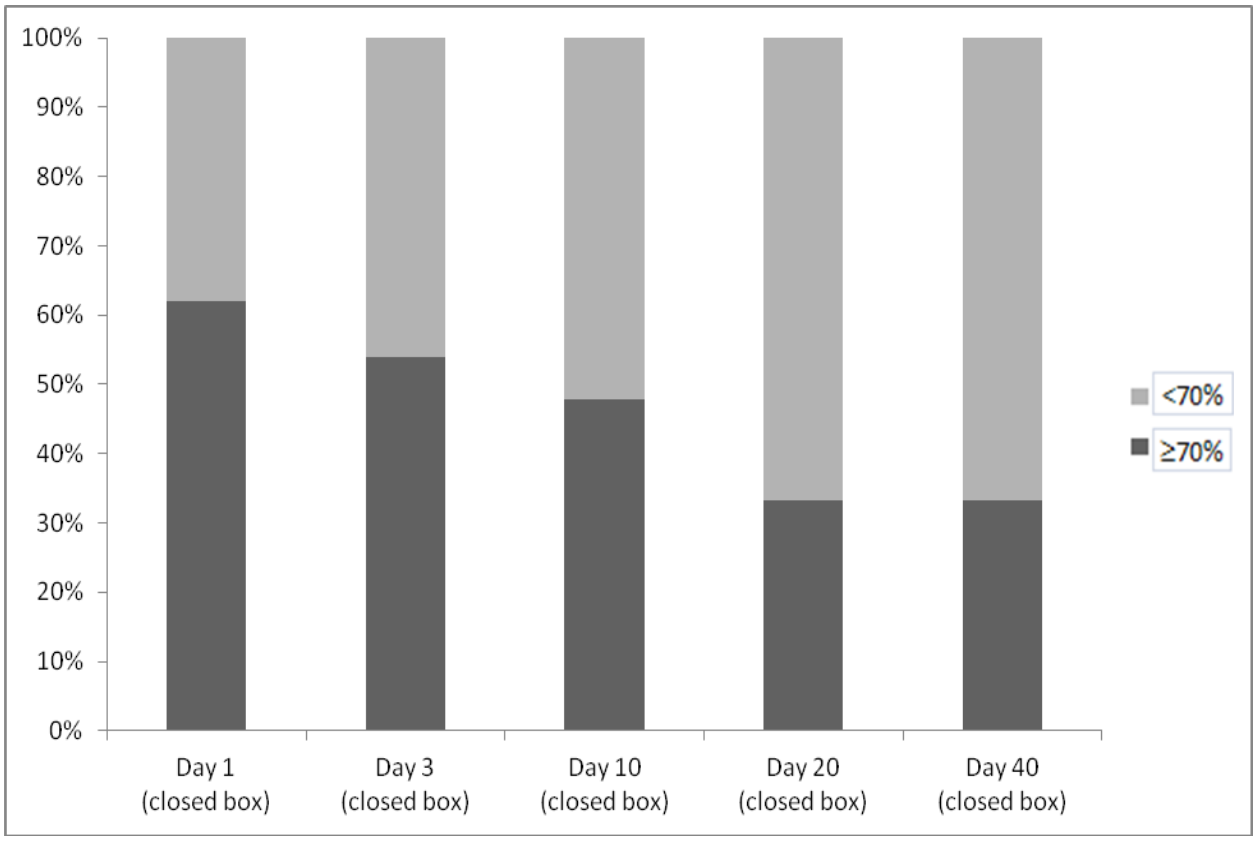

Figure 13: Percent STR profiles (AmpF*l*STR® Identifiler®) shown from volunteers' fingerprints stored at room temperature for the time indicated: 1, 3, 10, 20, and 40 days. Fingerprints were protected from dust in closed boxes. Profiles of ≥70% and <70% are presented in dark and light grey, respectively.

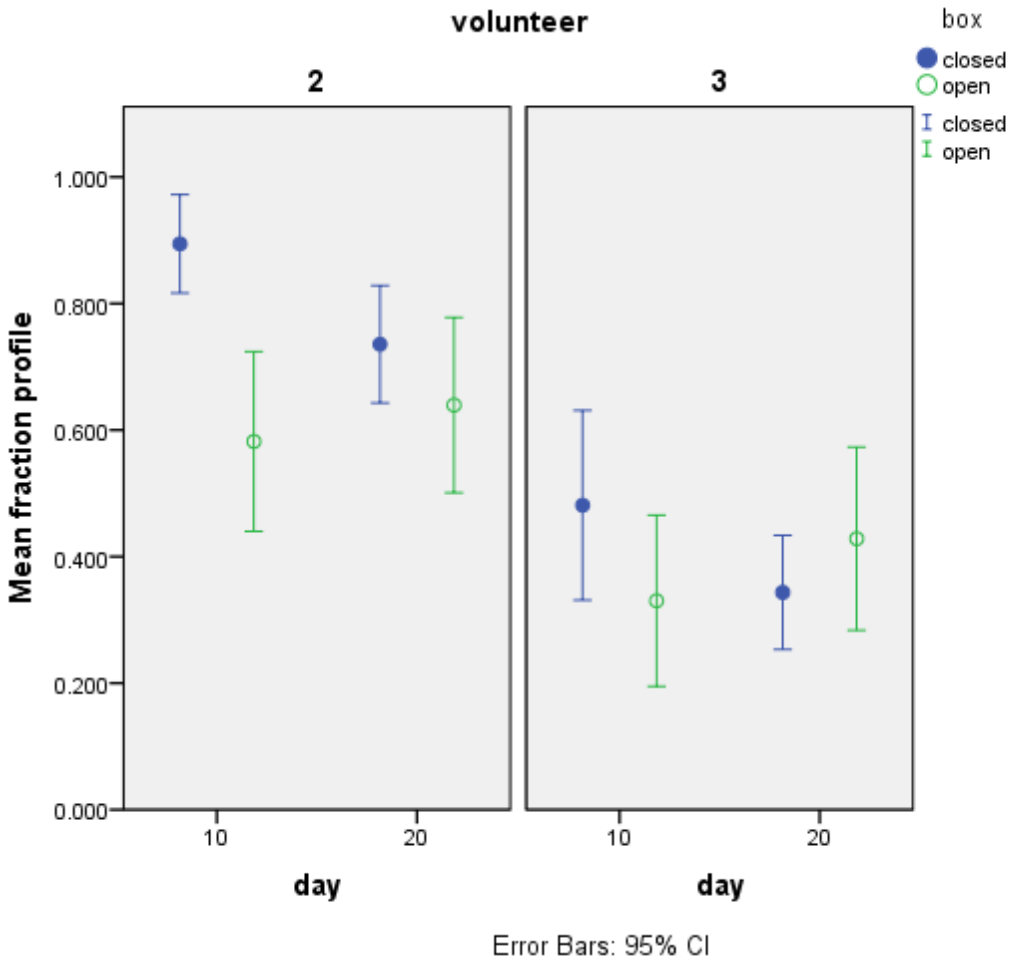

Figure 14: The mean fraction STR profiles (y-axis) shown from volunteers' fingerprints stored at room temperature for 10 and 20 days (x-axes). Fingerprints were protected from dust in closed boxes (filled circles in blue), or exposed to in-house conditions (open circles in green).

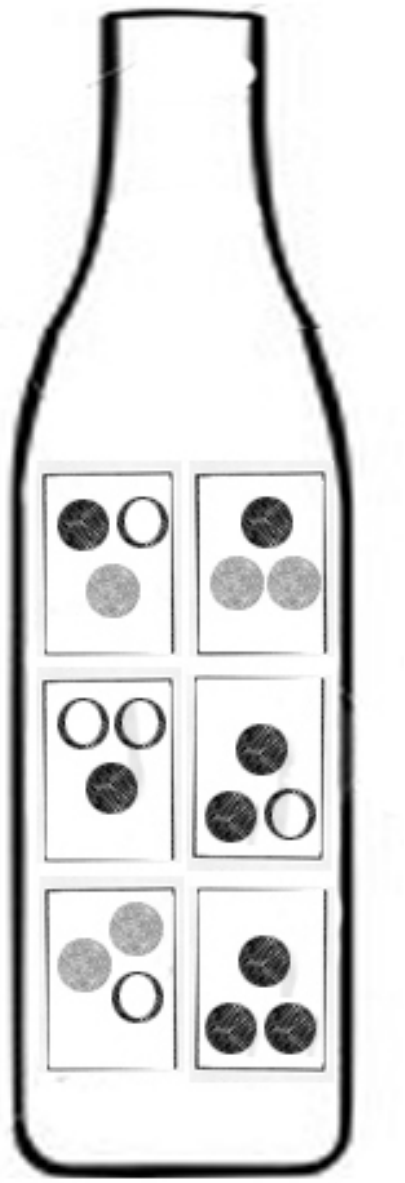

Figure 15: The trunk of a bottle was sectioned into six equal segments. The bottle was touched consecutively by three individuals.

Figure 16: Top: PALM system and laser ablation of a membrane slide on inverted microscope (pictures taken from (*61*)).
Bottom: Axiozoom and aureka® micromanipulator (*36*).

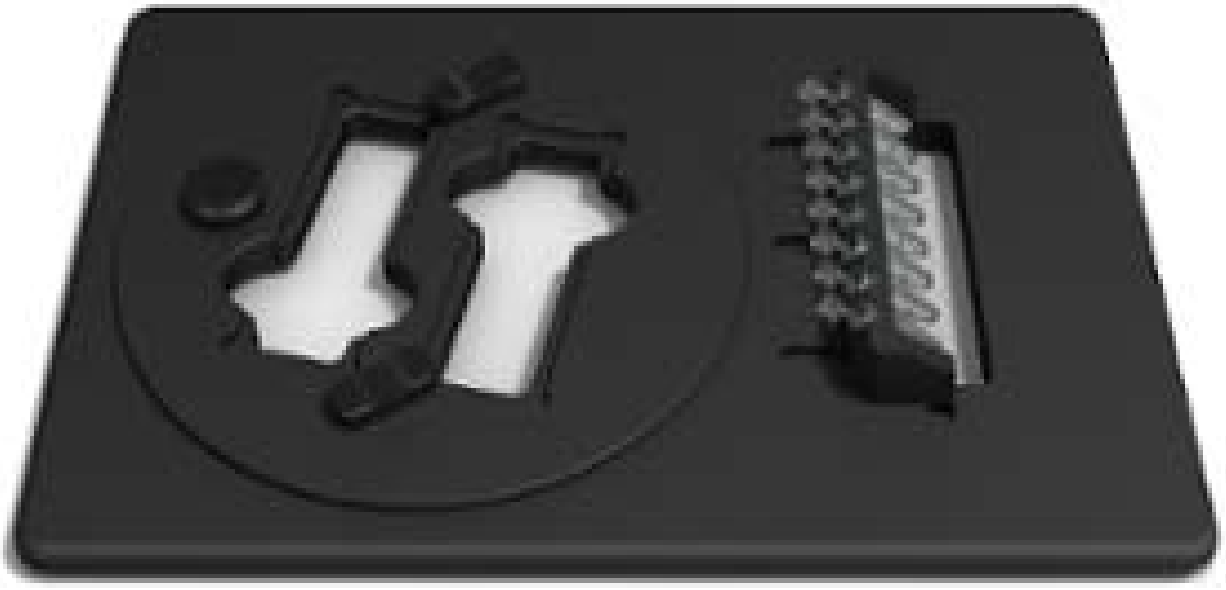

Figure 17: Axiozoom stage (designed by us): One slide contains the spheres, the other the cellular material of which single cells are picked, plus a rack for reaction tubes.

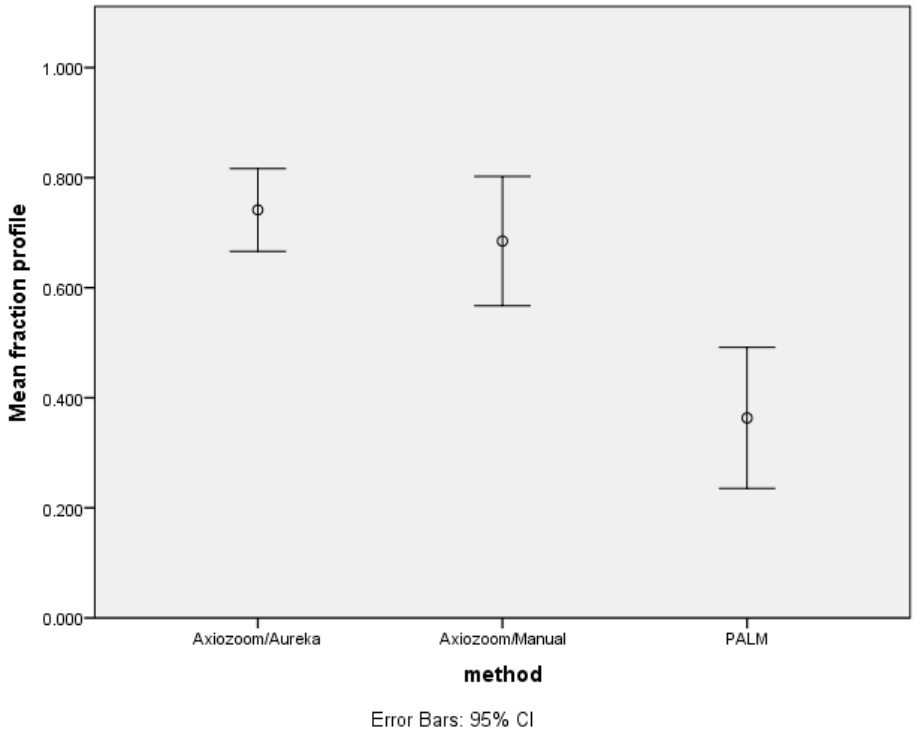

Figure 18: The mean fraction STR profiles (y-axis: AmpF/STR® Identifiler®) shown from 10 buccal cells collected by using the Axiozoom/Aureka, Axiozoom/Manual, and the PALM. The mean fraction was calculated from 25 repeats, the error bars show the 95% confidence interval (SPSS).

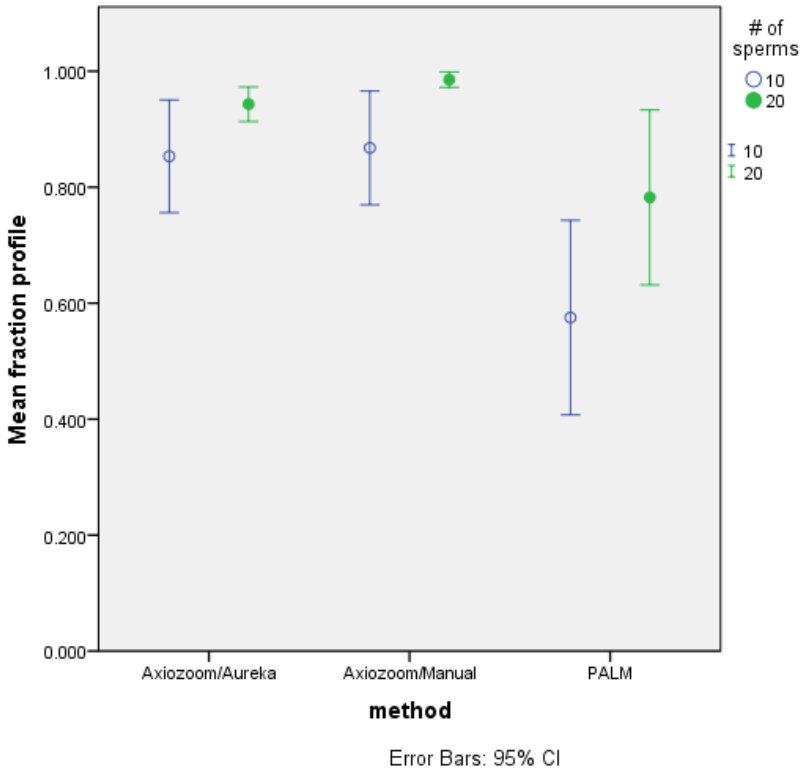

Figure 19: The mean fraction STR profiles (y-axis: AmpF/STR® Identifiler®) shown from 10 and 20 sperm cells (blue, open circles: 10 cells, green, filled circles: 20 cells) and the 95% confidence interval (error bars). (Repeats: 10 cells: Axiozoom/Aureka: 10, Axiozoom/Manual: 10, PALM: 9; 20 cells: Axiozoom/Aureka: 12, Axiozoom/Manual: 11, PALM: 10)

APPENDIX 1: Preparation of cells for micromanipulation

1.1 Preparation of epithelial cells (buccal)

- Collect buccal cells from volunteers by using a cotton swab to rub inside of their cheek(s).
- Smear the cells on the PEN membrane slide by softly rubbing the cotton swab on the membrane.
- Place a slide on a steam (above the boiling water) for few seconds to flatten the cells.
- Check slide under the microscope to ensure that cells were flattened and single cells are present. If lots of cell clumps are found, place slide on steam and use the same cotton swab to separate clumps and generate individual cells.
- Check slide under the microscope, if presence of single cells can be confirmed, place the slide on a thermobrite for 2 min at 56°C to fix the cells.
- Stain cells with NFR (mixture of 0.07M aluminum sulfate and 0.05% NFR) by adding few drops of NFR and incubate for 10 min at RT.
- Wash the stain completely off the slide by using dH_2O followed with 70% EtOH.
- Place the slide back on thermobrite for 2 min at 56°C for drying.
- Proceed with cell collection (Appendix 2).

1.2 Preparation of fingerprints

- Press the fingertip for a few seconds against glass slide, PEN membrane slide or on SIRCHIE tape.
- Proceed with cell collection (Appendix 2)

1.3 Preparation of sperm cells

- Use cotton swab to smear sperm cells on PEN membrane slide
- Check slide under the microscope. If presence of single cells is confirmed place the slide on a thermobrite for 2 min at 56°C to fix the cells.
- Stain cells with NFR (mixture of 0.07M Aluminum sulfate and 0.05% NFR) by adding few drops of NFR and incubate the slide for 10 min at RT.
- Wash the stain completely off the slide by using dH_2O followed with 70% EtOH.
- Place the slide back on a thermobrite for 2min at 56°C.
- Proceed with cell collection (Appendix 2).

APPENDIX 2: Collection of single cells – micromanipulation

2.1 Arabic gum to lift single epithelial and sperm cells

- Mix 2.75 ml of Arabic gum and 0.3 ml of glycerol and smear less than 1 ml of the mix onto a glass microscope slide that was previously cleaned in 10% bleach, followed by dH_2O and 70% EtOH and dried with Kim wipes.
- Dry overnight. If the mix is still liquid, place slide on a heating block (heated to no more than 60^{o}C) and inspect changing of the viscosity by stirring it with a micro-tool (Cricon Ultra Microlance, by McCrone Group, Westmont, Illionis, USA). Take the slide of the heating block when the Arabic gum/glycerol mix can easily be formed.
- Place slide under the Axiozoom V.16 microscope to inspect sphere preparation by using the Cricon Ultra Microlance microtool scoop a small portion of the mix and roll it to a sphere, then measure the size of the generated spheres. For single cell picking prepare spheres of 100-200 µm in diameter. Smaller spheres are more precise for collecting individual cells.
- Prepare, depending on the number of samples, spheres in advance. One sphere of ~ 200 µm in diameter can be used to collect up to 10 sperm cells or up to 5 buccal cells.
- Pick an Arabic gum/glycerol sphere with a tungsten needle. Make sure the sphere is firmly attached to the top on a needle.
- Pick cells manually by observing under the microscope.
- Release the sphere into the extraction buffer of a 0.2 ml tube.

2.2 Rubber cement for swabbing partial fingerprints

- Smear less than 1 ml of rubber cement onto a glass microscope slide that was previously cleaned in 10% bleach, followed by dH_2O and 70% EtOH and dried with Kim wipes.
- Rubber cement is viscous enough and can be immediately used to form spheres.
- Place slide under the Axiozoom V.16 microscope to inspect sphere preparation by using the Cricon Ultra Microlance microtool scoop a small portion of the Rubber cement and roll it to a sphere, then measure the size of the generated spheres. Approximately, one or two spheres, 200-300 µm in diameter, can be used to swab 1/6 of the fingerprint, depending on density of cells within the print.

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- Prepare sufficient spheres depending on the number of samples that will be generated.
- Pick the sphere with aureka tweezers and make sure the sphere protrudes.
- Observe collection under the microscope.

2.3 Cotton spheres to swab entire fingerprints

- Use Dumont N5 tweezers to pull out a few threads of cotton from a cotton swab.
- Moisten cotton threads with water, 5% Triton or extraction buffer. Remove the excess liquid by squeezing the cotton against a glass slide and by rolling.
- Use tweezers to roll the threads into a sphere.
- One sphere is sufficient for one fingerprint. If there is a lot cellular material, more spheres could be used to swab a print.
- Observe swabbing under the microscope.

2.4 Tape to lift fingerprints off larger objects

- Cut a piece of SIRCHE tape of about 7 cm
- Hold ends of tape and press the sticky side against the surface to lift the print.
- Lift the tape slowly by pulling it from one side.
- Place tape into metal frame and proceed with manual swabbing. Use a glass microscope slide below the frame to support the tape when performing the swabbing.
- To swab the tape, pull few threads from the cotton swab with manual Dumont N5 tweezers, moisten the swab with water, buffer or detergent, roll it into the sphere, and hold the sphere manually with tweezers to swab the sticky side of the tape.
- Perform swabbing under the Axiozoom V.16 microscope. Swab areas only where cells are detected.
- Cutting tape: use blade X-Acto knife or Feather surgical blade. Transfer of cuts to reaction tube manually with Dumont N5 tweezers.

APPENDIX 3: DNA extraction

3.1 DNA extraction from swabs of fingerprints and epithelial cells (ZyGem)

- Use *prep*GEM *Tissue* extraction kit (ZyGEM, Hamilton, NZ)
- Prepare extraction mixture per sample by adding:
    - 17.8 µl of DNA-free water,
    - 2 µl of 10x Buffer, and
    - 0.2 µl of *prep*GEM enzyme
- Prior to placing the samples on thermal cycler for incubation briefly spin the samples in the centrifuge (few seconds at approximately 1000 rpm).
- Incubate samples on thermal cycler at:
    - 75 °C for 15 minutes
    - 95 °C for 5 minutes followed by 4 °C until the samples are taken off

3.2 DNA extraction from sperm cells

- Add to each tube:
    - 200 µl of 5% Chelex (from a well-resuspended Chelex solution)
    - 1 µl of 20 mg/ml Proteinase K
    - 7 µl of 1 M DTT
- Use the pipette tip when adding the DTT to thoroughly mix the contents of the tubes.
- Incubate at 56°C for approximately 2 hours.
- Vortex at high speed for 10 to 30 seconds.
- Incubate at 100°C for 8 minutes using a screw down rack.
- Vortex at high speed for 10 to 30 seconds.
- Spin in a microcentrifuge for 2 to 3 minutes at 10,000 to 15,000 x g (13,200 rpm).
- Store the extracts at 2 to 8°C and/or submit for amplification.