Forensic Science International: Reports 4 (2021) 100240

Contents lists available at ScienceDirect

Forensic Science International: Reports

journal homepage: www.sciencedirect.com/journal/forensic-science-international-reports

Forensic Genetics

DNA profiling of single sperm cells after whole genome amplification

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ARTICLE INFO

Keywords:
Short tandem repeat (STR)
Forensic genetics
DNA typing
Whole genome amplification
Y-STR
X-STR
Micromanipulation
Sperm cell
Sexual assault

ABSTRACT

Intimate swabs taken for examination in sexual assault cases typically yield mixtures of sperm and epithelial cell types. While powerful, differential extraction protocols to overcome such cell type mixtures by separate lysis of epithelial cells and spermatozoa can still prove ineffective, in particular if only few sperm cells are present or if swabs contain sperm from more than one individual leading to complex low level DNA mixtures. A means to avoid such mixtures consists in the analysis of single micromanipulated sperm cells. However, the quantity of DNA from single sperm cells is not sufficient for conventional STR analysis. Here, we describe a simple method for micromanipulating individual sperm cells from intimate swabs and show that whole genome amplification can generate sufficient amounts of DNA from single cells for subsequent DNA profiling. We recovered over 80% of alleles of haploid autosomal STR profiles from the majority of individual sperm cells. Furthermore, we demonstrate that in mixtures of sperm from two contributors, Y-STR and X-STR profiles of individual sperm cells can be used to sort the haploid autosomal profiles to develop the diploid consensus STR profiles of the individual donors. Finally, by analyzing single sperm cells from mock sexual assault swabs with one or two sperm donors, we showed that our protocols enabled the identification of the unknown male contributors.

1. Introduction

DNA profiling of intimate swabs from sexual assault cases has always presented a challenge because if profiled directly this material would typically yield mixed DNA profiles originating both from epithelial (vaginal/anal/buccal) cells of the complainer and sperm cells from the perpetrator. Furthermore, the locations where such epithelial and sperm cell mixtures would typically be found (vagina/anus/mouth) are generally 'hostile environments' to sperm cells with such cells gradually being lost over a period of hours or days due to a combination of washing, natural biological processes and the simple action of gravity meaning that the proportion of DNA from sperm cells in such mixtures would rapidly decline. To help overcome this problem, differential lysis methods were developed that seek to create a sperm cell enriched fraction (the 'sperm fraction') and a fraction for all other cell types (the 'cellular fraction') [1]. These methods are based on a protocol that differentially lyses epithelial and sperm cells, taking advantage of the higher resistance of the sperm heads against the commonly used cell lysis buffer. Various modifications of this protocol have been developed, because effectiveness has been variable, warranting technical improvement (reviewed in [2]). Two major drawbacks of the differential lysis methods are (i) a considerable loss of sperm DNA which hampers analysis in cases where sperm numbers are low [3], and (ii) the inability of the method to separate the sperm of several contributors, e.g. in multiple rape cases or when there is also a legitimate partner. Although DNA mixture deconvolution has improved in recent years with the introduction of continuous interpretation methods [4], there are still cases whereby a lower amount of material or a higher number of

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https://doi.org/10.1016/j.fsir.2021.100240
Received 21 June 2021; Received in revised form 13 October 2021; Accepted 13 October 2021
Available online 16 October 2021

contributors go beyond safe interpretational limits.

As a physical means of mixture deconvolution of intimate swabs, the isolation of individual sperm cells has been evaluated in a forensic context, but has not yet been commonly applied to casework. Such casework applications could include the avoidance of mixtures, the expansion of 'time since intercourse' windows for successful profiling, and the manipulation of historic samples in cold case reviews. The methods for single cell isolation rely on a morphological identification of sperm cells by microscopy or by sperm-specific staining techniques that are compatible with subsequent DNA analysis. For the isolation of sperm cells, laser capture microscopy (LCM) has been successfully applied as well as micromanipulation using aspiration capillaries, adhesive microbeads or optical tweezers that are manually or semi-automatically controlled [5–10]. Other means of singling out individual sperm cells consist of microfluidic chambers or flow cytometry (reviewed in [2]).

With aspiration capillary-based micromanipulation, successful analysis of mitochondrial DNA of single sperm cells was demonstrated after two rounds of PCR [9]. Although the principal feasibility of STR analysis of single cells has previously been demonstrated [11], typically stochastic effects will hamper such analyses [12]. These effects are typically seen in analysis of small DNA amounts (referred to as low template DNA, LT-DNA) and include pronounced stuttering, strongly imbalanced profiles, allele drop-out (ADO) or allele drop-in (ADI) [13]. Using conventional STR analysis, minimal sperm numbers for obtaining complete profiles were reported to range between 20 and 50 sperm cells [5,7,10]. Thus, whereas the problem of sperm cell enrichment can be solved, the identification of contributors in multiple rape cases or cases also with partners remains a challenge. One idea has been to group the sperm cells of individual contributors from a mixture by Y-STR haplotyping. With the help of on-chip low volume PCR, diagnostic Y-STR loci were identified and could be used to assign sperm cells to individual donors and reconstruct their full autosomal STR profiles from the incomplete haploid STR profiles [14].

In the present paper we apply whole genome amplification (WGA) as a pre-amplification step to generate sufficient amounts of genomic DNA from single sperm cells for a subsequent conventional STR analysis. WGA methods are based on theoretically uniform exponential amplification of all regions of genomic DNA using short random primers and can be subdivided into two major classes, depending on whether they use either PCR or isothermic multiple strand displacement (MDA) amplification (reviewed in [15]). Further variations of the methods combine PCR- and MDA-like processes or amplify randomly generated genomic DNA fragments tailed with adaptor sequences [16–19]. There are several PCR-based protocols called Degenerate Oligonucleotide-Primed PCR (DOP), Primer-Extension Preamplification (PEP) and variants thereof, as well as several variations of the MDA method that have been tested in forensic analysis of LT-DNA [19–26]. So far results were mostly disappointing with sensitivity often not exceeding current multiplex PCR kits, and profiles displayed pronounced stochastic effects, in particular high stuttering, strongly imbalanced profiles, ADO and ADI. For haploid profiles such as from single sperm cells, however, locus imbalance may pose less of a problem, and thus we reasoned that WGA might be suited to pre-amplify DNA from single sperm cells for subsequent forensic STR analysis. Due to medical interests in single cell diagnostics [27], several novel commercial WGA kits are optimized for single cell analysis but none has yet been tested in a forensic context. In a preliminary study we identified the MDA-based Repli-g single cell kit as a promising candidate for single sperm STR typing [28]. By applying a micromanipulation technique using tungsten needle tips covered with adhesive [29], we show that using Repli-g WGA haploid STR profiles from single sperm cells can be obtained with a high success rate. Furthermore, we use Y-STR and X-STR haplotyping of single sperm cells to develop the diploid autosomal donor consensus STR profiles of mixtures of two sperm donors and successfully analyze mock sexual assault swabs spiked with female epithelial cells and semen of one or two donors.

2. Materials and methods

2.1. Biological material and mock sample preparation

To perform the experiments described in this research project, volunteers were invited to donate their semen and/or buccal swab. Each volunteer provided a signed consent for their participation. Ethical approval of the study was granted by the Robert Gordon University's Ethics Review Panel. Collected semen was stored at −20 °C. All mouth swabs were air-dried and stored at −20 °C. Neat semen from two donors was used for testing with WGA and autosomal STRs as proof of principle. Furthermore, neat semen was used for testing with WGA combined with X-, and Y-STRs. Mock intimate swabs were created by soaking the head of mouth swabs from female donors with 100 µl 1:50 diluted semen from one or from two donors in an eppendorf tube. Based on rough estimations each swab was thus spiked with approximately 200,000 sperm cells. Spiked mock intimate swabs were air dried at room temperature and stored at −20 °C.

2.2. Extraction of sperm cells from swabs

To release sperm cells from the cotton swabs, frozen mock swabs were thawed at room temperature for 30 min. Then, with a scalpel the cotton part was cut off from the swab's end on a DNA free glass petri dish and split in half. With a pair of tweezers each cotton half was transferred in a microcentrifuge tube (1,5 ml). Then 500 µl of buffer ATL and 25 µl of Proteinase K from QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was added to each tube in order to lyse non-sperm cells. Afterwards, the tubes were vortexed for 10 s and then incubated in a thermoshaker for 30 min at 56 °C with 900 rpm shaking. The tubes were vortexed again for 10 s and incubated for 30 min at 56 °C with shaking (900 rpm). Tubes were then vortexed for 10 s and briefly spun down. Next, each cotton part was put into a pluriStrainer Mini 10 µm cell strainer (pluriSelect Life Science,Leipzig, Germany) with 10 µm pore size that was placed back into the tubes and centrifuged at 14,000 g for 5 min to release remaining liquid with spermatozoa left on the cotton parts. The cell strainer containing the cotton part and the supernatant, were discarded without disturbing the pellet. Then 500 µl 1x PBS was added to the tubes and pellets were re-suspended by tapping and pulse-vortexing. Afterwards, the cells were pelleted again by centrifuging at 14.000 g for 5 min and the supernatant was discarded without disturbing the pellet. The pellet was resuspended in 300 µl of DNA free water by tapping and pulse-vortexing. Then, tubes were spun down briefly to remove any liquid on the top of the tube and lid. Afterwards, 20 µl of cell suspension was placed on a microscope slide and analyzed with a light microscope at 200× magnification. In case when the suspension was highly concentrated with sperm cells, the suspension was diluted with DNA free water to sufficiently separate the sperm cells.

2.3. Micromanipulation of sperm cells

During micromanipulation, the operator was wearing gloves and a face mask, and all pipetting steps before and after micromanipulation were performed in a safety cabinet. Single spermatozoa were isolated by spreading out 100–300 µl of cell suspension onto petri dishes containing thin layers of 1% agarose gel, aiming at a number of approximately 50–100 sperm cells per petridish. To these ends, agarose was suspended in deionized water, dissolved by boiling in a microwave oven and poured into 90 mm petridishes until the surface was covered by a thin (approximately 3 mm) layer, and allowed to solidify by cooling down. Hereby, the petridish was covered with a slightly lifted lid to prevent airborne contaminations, but to allow for drying. After drying for 30 min, micromanipulation was performed by placing the agarose gel petri dish containing the spermatozoa underneath an inverted microscope (Axiovert S 100, Zeiss, Oberkochen, Germany) equipped with a TransferMan micromanipulation system (Eppendorf, Hamburg, Germany) and the

search for spermatozoa was carried out at 200× magnification. A small amount of adhesive was collected from a piece of 3 M tape (3 M, Saint Paul, USA) with a tungsten needle with a tip diameter of 5 μm (PLANO GmbH, Wetzlar, Germany). With the adhesive at the tip of the needle, a single spermatozoon was picked from the agarose gel surface and then transferred into a PCR tube containing 4 μl DNA free water by swirling the tip of the needle in the water for 10 s. Tubes containing isolated single sperm cells were then subjected to whole genome amplification with REPLI-g Single Cell WGA kit (Qiagen, Hilden, Germany).

2.4. DNA extraction and WGA

Genomic DNA (gDNA) from buccal cells taken with cotton swabs was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Lysis of sperm cells occurred during the lysis step of the REPLI-g single cell WGA kit (Qiagen, Hilden, Germany) that was carried out following the manufacturer's protocol. WGA products were cleaned up with innuPREP PCRpure Kit (Analytik Jena, Jena, Germany) following the manufacturer's protocol, however using 450 μl Binding Buffer and repeating the first centrifugation step with centrifugation at 12,000 g for 2 min.

2.5. STR analysis

All PCR amplification cycles were carried out with a GeneAmp PCR System 9700 thermocycler (ThermoFisher, Waltham, USA). Genomic DNA was quantitated photometrically using a NanoPhotometer (Implen, Munich, Germany). For autosomal STR analyses, WGA products were not quantitated. For analysis of gonosomal STR loci, WGA products were quantitated using the Investigator Quantiplex Pro Kit (Qiagen, Hilden, Germany) on a 7500 Fast Real-Time PCR System (ThermoFisher, Waltham, USA) according to the manufacturer's instructions and adjusted to 1 ng/μl for X-STR analysis, or 2,5 ng/μl for Y-STR analysis.

For autosomal STR loci and Amelogenin, 1 ng of gDNA or 1 μl of 1:50 diluted WGA product was analyzed using the PowerPlex ESX17 kit (Promega, Madison, USA). The 1:50 dilution was empirically determined to be suited for subsequent analysis and was necessary to comply with the high amounts of DNA synthesized. PCR reactions were carried out in 5 μl total reaction volume and amplified with 1 cycle of 96 °C for 2 min, 30 cycles of 94 °C for 30 s, 59 °C for 2 min and 72 C° for 1.5 min; followed by 45 min at 60 °C and cooling at 4 °C.

For analysis of X-STR loci, 1 μl of 1 ng/μl gDNA or of 1 ng/μl WGA product was subjected to PCR using the Investigator Argus X12 QS kit (Qiagen, Hilden, Germany). Reactions were carried out in 5 μl total reaction volume and amplified with 1 cycle of 94 °C for 4 min, 5 cycles of 96 °C for 30 s, 63 °C for 120 s and 72 °C for 75 s, followed by 25 cycles of 94 °C for 30 s, 60 °C for 120 s and 72 °C for 75 s, and one final extension at 68 °C for 60 min.

Y-STR loci were amplified from 2 μl of 2,5 ng/μl gDNA or of 2,5 ng/μl WGA product using PowerPlex Y23 kit (Promega, Madison, USA). Reactions were carried out in 10 μl total reaction volume and amplified with 1 cycle of 96 °C for 2 min, 30 cycles of 94 °C for 10 s 61 °C for 1 min, 72 °C for 30 s, followed by a terminal extension at 60 °C for 20 min.

2.6. Fragment analysis by using capillary electrophoresis

PCR products were analyzed by capillary electrophoresis on an ABI Prism 310 or ABI Prism 3130 Genetic Analyzer (ThermoFisher, Waltham, USA). A volume of 1 μl product was denatured in 12 μl deionized HiDi™ formamide (ThermoFisher, Waltham, USA) and 0,5 μl DL500 ORN size standard (Serac GmbH, Bammental, Germany), WEN ILS 500 (Promega, Madison, USA) or Biotype SST-BTO 60–500 bp (Biotype, Dresden, Germany) at 95 °C for 4 min. A volume of 1 μl of 1:50 diluted autosomal STR PCR products from WGA products as well as Y-STR PCR products were denatured as described above. A volume of 1 μl of 1:100 diluted X-STR PCR products was denatured as described above. Denatured samples were injected at 3kV for 3 s. Data was genotyped with GeneMapper™ v3.0 (Thermo Fisher, Waltham, USA) with the peak amplitude threshold for allele calling set to 50 RFU's.

Allele recovery was calculated by dividing the number of alleles recovered in the sample by the total number of alleles from the reference sample multiplied by 100 to achieve the percentage. For calculating allele recoveries of haploid autosomal STR profiles of sperm the number of alleles recovered was divided by 17 (the number of loci analyzed).

3. Results

3.1. Micromanipulation of sperm cells

In the initial experiments using aspiration capillaries to isolate single sperm cells, success rates of STR profile recovery after Repli-g single cell WGA kit (hereafter shortly termed Repli-g WGA) were variable. Because the transfer of sperm cells from the capillary to the reaction vial as well as the concomitant transfer of liquid was difficult to control, we switched to a micromanipulation technique that uses a tungsten needle the tip of which had been coated with water-soluble adhesive to pick individual sperm cells [29]. Before doing so, the compatibility of the water-soluble adhesive with cell lysis and WGA reactions was experimentally verified. In addition, we tested suitability of several substrata for sperm immobilization and subsequent picking. Among those, polyethylene terephthalate, polycarbonate, polyethylene, polymethyl methacrylate, adhesive tape, polystyrene petri dishes or non-adherent cell culture flasks, WD40- or Repel-silane-coated microscopic slides all resulted in strong sticking of the sperms and their subsequent disintegration during picking. In contrast, 1% agarose gels proved compatible with sperm picking and were thus used in the following experiments. Thin agarose gels are translucent, enabling microscopic identification of sperm cells, and cells are sufficiently immobilized without too firm adherence. Picking of sperm cells was visually controlled under the microscope. Transfer of the picked cell into the reaction vial could not be visually controlled and was inferred from the complete dissolving of the adhesive in the lysis buffer which was subsequently confirmed by microscopic inspection of the needle tip.

3.2. Single sperm analysis

Using the tungsten needle-based micromanipulation, 18 individual sperm cells from two known donors were isolated from neat semen and subjected to Repli-g WGA and subsequent STR analysis. Generated WGA products were used for analyzing autosomal STRs, as well as X- and Y-STRs. At best, autosomal STR typing of each sperm cell would show a haploid DNA profile. However, in X- and Y-STR typing, only one of the two profiles would be obtained since each spermatozoon will carry either an X-chromosome or a Y-chromosome.

The PCR product from most samples was diluted by 1:50 to avoid oversaturating the capillary electrophoresis. A few samples had to be further diluted to 1:100 because the amount of PCR product of some alleles was still too high. Since higher dilution sometimes results in ADO, both undiluted and diluted PCR products per sample were analyzed and allele recovery was defined as the consensus recovered peaks from the two preparations (exemplified in Section 3.3, Fig. 3). To determine allele recoveries, consensus profiles were compared to STR profiles of the donors generated from buccal swabs without the help of WGA. Haploid autosomal STR allele recovery from WGA products ranged from 18% to 100% (Table 1), with most sperm cells displaying over 80% haploid allele recovery and 3 sperm cells showing full haploid profiles in their individual consensus profiles. WGA products were positive for amelogenin and showed either AMELX or AMELY as expected. The electropherograms from successfully amplified autosomal STR loci showed no evidence of multiple sperm cells being typed as only one of the two possible alleles of each STR marker was present (Fig. 1).

Next, WGA products from the single sperm cells were subjected to X-

Table 1
Allele recoveries of single sperm cells after WGA.

			Allele recovery (%)		
Donor	*Sample*	*AMEL*	*A-STRs*	*Y-STRs*	*X-STRs*
A	gDNA*	XY	100	100	100
	Sperm 1	Y	18	100	0
	Sperm 2	Y	88	91	0
	Sperm 3	Y	88	95	0
	Sperm 4	Y	82	82	0
	Sperm 5	Y	29	68	0
	Sperm 6	Y	82	68	0
	Sperm 7	X	100	0	91
	Sperm 8	X	82	0	82
	Sperm 9	X	94	0	73
	Sperm 10	X	100	0	64
	Sperm 11	X	82	0	91
B	gDNA	XY	100	100	100
	Sperm 1	Y	94	91	0
	Sperm 2	Y	94	91	0
	Sperm 3	Y	88	91	0
	Sperm 4	Y	65	68	0
	Sperm 5	Y	100	100	0
	Sperm 6	X	94	0	100
	Sperm 7	X	76	0	91

* gDNA, genomic DNA from buccal swab, no WGA.

and Y-STR profiling. As summarized in Table 1, all WGA products from single sperm cells which were positive for AMELY but negative for AMELX showed Y-STR alleles with no X-STR alleles detected. Conversely, AMELX-positive sperm cells did not reveal Y-STR profiles, but X-STR profiles. Examples of electropherograms are shown in Fig. 2. Recovery rates of Y-STR alleles ranged between 68% and 100% for the 6 or 5 AMELY-positive sperm cells from either donor. For X-STRs, allele recoveries ranged between 71% and 93% for the 5 sperm cells of one donor and was 93% for the two AMELX-positive sperm cells of the other donor. Of note, two sperm cells of donor A were not concordant with the donor alleles, allele 18 of DXS10074 possibly due to a −1 slippage mutation of allele 9, allele 14.1 of DXS10135 to be considered as ADI (Table 2).

Because there is no recombination in the male germ line between the parts of the Y- and X-chromosomes bearing the gonosomal STR markers, both Y-STR and X-STR profiles are haplotypes and allow the assignment to individual donors (if not closely paternally related) with the help of diagnostic alleles that are specific for either donor. By this means it is possible to deconvolute a mixture of sperm cells of two donors. As a proof of principle, we identified diagnostic gonosomal STR alleles (Table 2) and used them to establish the consensus diploid autosomal STR profiles from the two sperm donors. As shown in Table 3, diploid STR profiles could be established for both donors, except for donor B's FGA locus, where none of the seven sperm cells analyzed contained the second allele. Furthermore, sperm 2 from donor B displayed both D3S1358 alleles, and this locus was thus excluded from the calculation

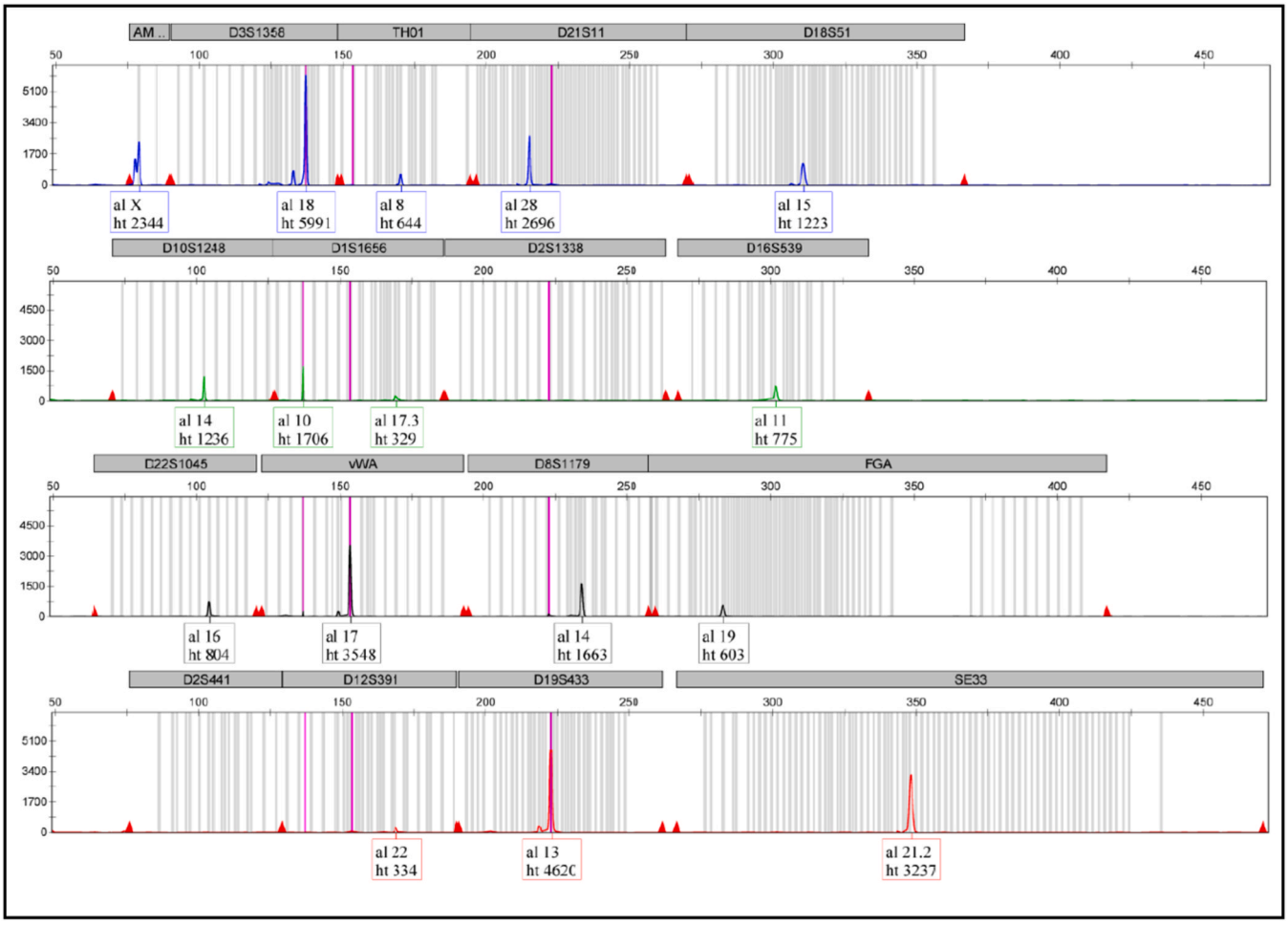

Fig. 1. Electropherogram of an autosomal STR profile from a single sperm cell. Genomic DNA was amplified with REPLI-g based WGA followed by STR analysis using PowerPlex ESX17. Shown is the electropherogram of an undiluted PCR product. Positions of potential bleed-through signals are indicated with a purple line. D1S1656 allele 10 has been wrongly called due to a bleed-through from D3S1338 allele 18. This is suggested by the unusual slim shape of the peak base.

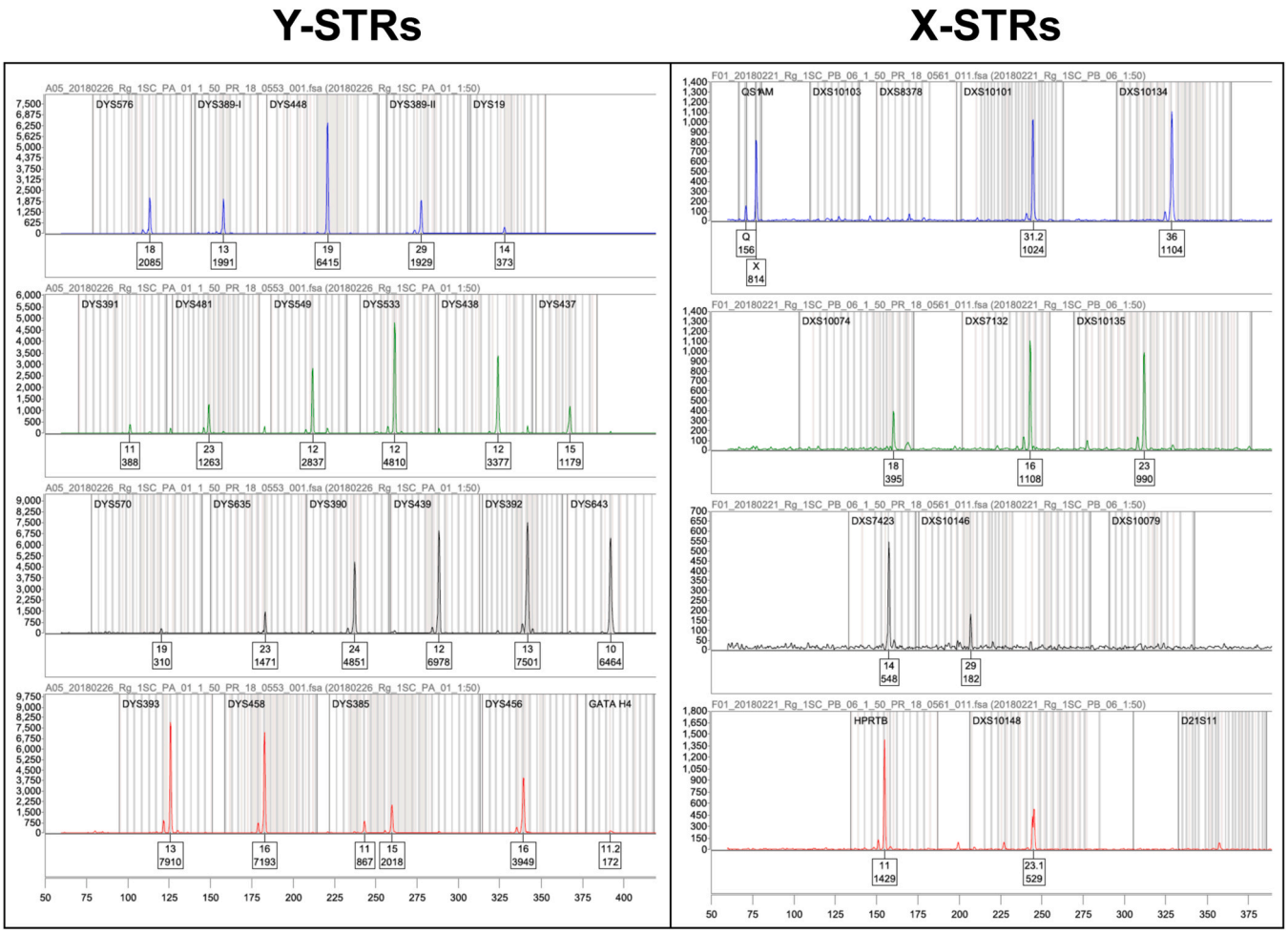

Fig. 2. Representative electropherograms of gonosomal STR profiles from single sperm cells. Genomic DNA was amplified with REPLI-g based WGA followed by Y-STR analysis using PowerPlex Y23 (left panel) or X-STR analysis using Investigator Argus X12 QS (right panel). Shown are electropherograms of 1:50 diluted PCR products.

of allele recoveries (Table 1).

3.3. Mock swabs with one male contributor

To address whether the micromanipulation works with semen dried on a cotton swab and to test the whole method in a more realistic scenario, mock sexual swabs were prepared by spiking cotton swabs with buccal epithelial cells of six different female donors and semen from either one or two known men. Additionally, mock sexual swabs spiked with semen from either one or two unknown men were prepared for validation of the method. In total there were six sperm donors, of whom a set of reference STR profiles was established from mouth swabs and compared to the autosomal and gonosomal STR profiles obtained from the mock swabs. All mock sexual swabs were prepared with semen dilutions appropriate to create sperm cell concentrations equivalent to a 1+/2+ slide sperm count as per Willott and Allard 1982 [30].

Results from experiments with mock swabs are summarized in Table 4. Derivation of a haploid consensus STR profile of one sperm of a mock sample is shown in Fig. 3. From the first swab (female P, male A) 10 sperm cells were isolated of which 5 could be successfully typed. Aiming at a minimum number of 3 sperm cells for identification of the donor, we thus decided to pick 7 sperm cells from the remaining swabs with single male contributors and 14 sperm cells from swabs with two male contributors.

As shown in Table 4, generally the numbers of successfully typed sperm cells differed between the swabs, and also the allele recoveries were on average lower than for sperm cells from neat semen. This might be due to the different storage conditions or treatments, e.g. the drying of cells or proteinase K treatment. For swabs with known single male contributors, all successfully typed sperm cells of swabs contained alleles matching the male contributors' reference profiles. Only in one of these swabs were ADIs observed and these could be attributed to the female contributor's profile, which might indicate a possible picking up and amplification of female cell-free DNA or DNA-containing cellular debris. ADIs also occurred in X-STR typing in two cases, and in Y-STR typing of one of the sperm cells. However, X-STR-positive sperms never were positive for Y-STRs and vice versa which, together with the fact that none of the autosomal loci displayed more than one allele, is consistent with the assumption that DNA of single cells was analyzed.

Two swabs with unknown single male contributors were tested as well. All successfully typed sperm cells contained alleles that matched the male contributor's reference profile, and it was possible to identify the unknown contributors. From one swab, however, a profile was obtained only from one spermatozoon out of seven. The autosomal haploid allele recovery of this spermatozoon was 56% (Table 4). Despite the low success rate, the allele recovery was high enough to match the correct reference profile. Also with the other swab, with a success rate of 4 out of 7 typed sperm cells, it was possible to match the correct reference profile. One STR locus of one of these sperm cells showed one ADI that was 1 repeat different from the reference allele number. Since no second allele from the same locus was seen, we propose that the ADI resulted from a slippage mutation.

Table 2
Diagnostic alleles of gonosomal STR loci.

		Alleles of diagnostic markers[2]														
		Y-STRs					*X-STRs*									
donor	*sample*	DYS576	DYS481	DYS570	DYS439	DYS385	DXS8378	DXS10101	DXS10134	DXS10074	DXS7132	DXS10135	DXS7423	DXS10146	HPRTB	DXS10148
A	gDNA[1]	18	23	19	12	15	11	29.2	37	19	13	29	15	30	12	25.1
	Sperm 1	18	23	19	12	15	–	–	–	–	–	–	–	–	–	–
	Sperm 2	18	23	–	12	15	–	–	–	–	–	–	–	–	–	–
	Sperm 3	18	23	19	12	15	–	–	–	–	–	–	–	–	–	–
	Sperm 4	18	23	19	12	15	–	–	–	–	–	–	–	–	–	–
	Sperm 5	18	23	–	12	–	–	–	–	–	–	–	–	–	–	–
	Sperm 6	18	23	19	–	15	–	–	–	–	–	–	–	–	–	–
	Sperm 7	–	–	–	–	–	11	29.2	37	19	13	29	15	–	12	25.1
	Sperm 8	–	–	–	–	–	–	29.2	37	19	–	29	15	30	12	25.1
	Sperm 9	–	–	–	–	–	–	–	37	18	13	–	15	30	12	25.1
	Sperm 10	–	–	–	–	–	–	29.2	37	19	13	–	–	30	–	25.1
	Sperm 11	–	–	–	–	–		29.2	37	–	13	14.1	15	30	12	25.1
B	gDNA	19	22	17	11	11,14	12	31.2	36	18	16	23	14	29	11	23.1
	Sperm 1	–	22	17	11	11,14	–	–	–	–	–	–	–	–	–	–
	Sperm 2	–	–	17	11	11,14	–	–	–	–	–	–	–	–	–	–
	Sperm 3	19	22	–	–	11,14	–	–	–	–	–	–	–	–	–	–
	Sperm 4	19	22	17	11	–,14	–	–	–	–	–	–	–	–	–	–
	Sperm 5	19	22	17	11	11,14	–	–	–	–	–	–	–	–	–	–
	Sperm 6	–	–	–	–	–	12	31.2	36	18	16	23	14	29	11	23.1
	Sperm 7	–	–	–	–	–	–	31.2	36	18	16	23	14	29	11	23.1

[1] *gDNA, genomic DNA from buccal swab, no WGA*

[2] *discordant alleles are highlighted in grey*

3.4. Mock swabs with two male contributors

Mock swabs containing sperm cells originating from two male donors were also used to test the described protocol for applicability to sexual assault cases in which the victim was sexually assaulted by two men.

At first one swab with two known male contributors, male E and male F, was tested. Here, 14 sperm cells were isolated of which 12 generated STR profiles (Table 4). Four haploid autosomal STR profiles matched the reference profile of male E and the other 8 matched male F. Furthermore, X-STR profiles were obtained from 6 sperm cells, however, Y-STR profiles were obtained from only 5 of the remaining sperm cells. All obtained Y- and X-STR profiles matched the correct male reference profiles. ADIs were only noticed in two of the X-STR profiles where in each profile a single ADI was observed. One of the ADIs was one repeat unit longer than the reference allele and thus presumably represented a slippage mutation. The other ADI differed by 3 repeat units.

Two swabs, each with two unknown male contributors were analyzed. From one swab an autosomal STR haploid profile from 12 of the 14 isolated sperm cells was obtained (Table 4). The success rate of the other swab was slightly lower, with 10 out of 14 successfully typed single sperm cells. From both swabs autosomal STR profiles as well as X- and Y-STR profiles matched the correct reference profiles. Both male contributors of the second swab were identified by comparing the 10 autosomal haploid profiles to the reference profiles. Two of the haploid STR profiles matched with male A and the other 8 matched with male F. From the second swab, however, all obtained autosomal STR profiles as well as the gonosomal STR profiles matched one and the same reference profile, male E, and the second contributor could not be identified. For the preparation of the two-donor swabs the same volume of semen from each male donor was used, however, sperm counts were not taken into consideration and we propose that unequal sperm counts of the two donors might be the reason that apparently only one male was sampled.

4. Discussion

Low numbers of sperm cells being overwhelmed by abundant epithelial cells and mixtures of sperm cells have long been challenges in the clarification of sexual assault cases often leading to uninterpretable trace level DNA mixtures or a total failure to profile the target component, and remain so even in the modern context of DNA interpretation software and Y-STR profiling.

In this paper we have established a method for STR analysis of single sperm cells that combines a simplified micromanipulation protocol and whole genome amplification, followed by conventional STR analysis. We show that it is possible to analyze STR profiles of single sperm cells and that these can be combined to establish diploid profiles of individual contributors. As an important outcome, our study thus demonstrates that increasing the analytical sensitivity using WGA is a viable option for forensic DNA profiling if applied to single sperm cells.

Of note, vaginal swabs after an assault are likely to impose additional problems (such as adsorption of victim DNA to sperm cells and impaired integrity of the sperm cells) [31]. Thus a higher number of sperm cells may be required in order to obtain samples for a successful analysis, both in terms of DNA integrity and in terms of admixed female DNA. Using artificially prepared swabs we showed that dried semen on cotton swabs is compatible with the described procedure. Thus, although more work would be required to define exact parameters for casework use, we would envisage this protocol to be part of a Forensic Scientist's 'toolbox' when investigating sexual offense cases. For example, the protocol could be considered if case information suggested the involvement of multiple perpetrators, if the 'time since intercourse' to the medical examination was at the far end of the plausible DNA profiling window, or if the complainer had repeatedly washed themselves. In such instances, the scientist might consider selecting one or more of the available intimate swabs and committing all of the swab head(s) for extraction and application to an agar preparation so that if any sperm cells were available

Table 3
Reconstruction of the diploid autosomal STR profiles of donor A and B from single sperm analysis.

donor	*sample*[a]	D3S1358		TH01		D21S11		D18S51		D10S1248		D1S1656		D2S1338		D16S539		D22S1045		vWA		D8S1179		FGA		D2S441		D12S391		D19S433		SE33	
A	**gDNA**[b]	**a**[c]	**b**	**a**		**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**		**a**	**b**	**a**		**a**	**b**	**a**	**b**	**a**		**a**	**b**	**a**	**b**	**a**	**b**
	Sperm 1			a						a																							
	Sperm 2	a		a			b				b	a			b	a		a		a		a			b	a		a		a			
	Sperm 3		b	a			b		b	a			b		b	a			b	a		a						a			b		
	Sperm 4	a		a		a		a		a				a		a				a		a		a						b		b		b
	Sperm 5								b	a													b											
	Sperm 6		b	a			b	a		a		a		a					b	a			b		b					b	a			
	Sperm 7	**a**		**a**		**a**		**a**		**a**			**b**	**a**		**a**			**b**	**a**		**a**			**b**	**a**				**b**		**b**		**b**
	Sperm 8		b	a		a			b		b		b	a					b			a				a		a			b	a		
	Sperm 9	a		a		a		a			b	a		a		a		a		a		a		a		a					b			b
	Sperm 10		**b**	**a**		**a**			**b**		**b**		**b**	**a**		**a**			**b**	**a**		**a**			**b**	**a**		**a**			**b**	**a**		
	Sperm 11		b			a		a		a				a		a			b	a		a			b	a		a						b
	consensus	a	b	a		a	b	a	b	a	b	a	b	a	b	a		a	b	a		a	b	a	b	a		a	b	a	b	a	b
B	**gDNA**	**a**	**b**	**c**	**d**	**c**	**d**	**a**	**d**	**c**		**c**	**b**	**c**	**b**	**c**		**c**		**c**		**a**	**d**	**c**	**d**	**c**	**d**	**c**		**c**		**c**	**d**
	Sperm 1	a			d	c			d	c				c		c		c		c			d	c		c		c		c			d
	Sperm 2	a	b	c			d		d	c			b		b			c		c			d	c		c		c		c			d
	Sperm 3		b				d	a		c			b	c		c		c		c			d				d	c		c		c		
	Sperm 4	a				c		a		c			b	c		c		c				a						c						
	Sperm 5	**a**			**d**	**c**		**a**		**c**		**c**		**c**		**c**		**c**		**c**		**a**		**c**		**c**		**c**		**c**		**c**		
	Sperm 6		b		d		d	a		c			b		b	c		c		c				c		c		c		c		c		
	Sperm 7		b	c				a		c			b					c		c		a		c						c				d
	consensus	a	b	c	d	c	d	a	d	c		c	b	c	b	c		c		c		a	d	c		c	d	c		c		c		d

[a] Sperm cells with complete haploid profiles are in bold letters.
[b] gDNA, genomic DNA from a buccal swab, no WGA.
[c] Allele numbers of each locus are symbolized with alphabetical letters a, b, c, d. If both donors have the same allele at one locus, the same letter is used. Between different loci of the same donor, identical letters do not represent identical allele numbers.

Table 4
Success rates of single sperm typing of mock swabs.

Contributors			*No. of sperms isolated*	*No. of successfully typed spermatozoa*			*Average allele recovery (%) ± standard deviation (%)*[a]			*No. of spermatozoa with ADI*		
Female	*Male 1*	*Male 2*		*A-STRs*[b]	*X-STRs*	*Y-STRs*	*A-STRs*[c]	*X-STRs*[c]	*Y-STRs*	*A-STRs*	*X-STRs*	*Y-STRs*
P	A	–	10	5 (4)	3	2	54 ± 27	83 ± 9	29 ± 28	0	1	0
Q	B	–	7	7 (5)	4	3	47 ± 12	69 ± 13	49 ± 7	0	1	0
R	C	–	7	2 (1)	0	1[d]	56 ± 44	–	61	1	–	0
S	D	–	7	2 (2)	2	0	75 ± 9	67 ± 23	–	0	0	–
P	unknown	–	7	1 (1)	0	1	56	–	52	0	–	0
Q	unknown	–	7	4 (4)	1	3	63 ± 14	83	73 ± 2	0	0	1
T	E	F	14	12 (7)	6	5	49 ± 18	67 ± 28	51 ± 14	0	2	0
U	unknown	unknown	14	12 (11)	4	8	71 ± 15	85 ± 5	71 ± 17	1	0	0
T	unknown	unknown	14	10 (6)	7	3	52 ± 21	69 ± 14	52 ± 23	0	1	0

[a] of successfully typed spermatozoa; percent values rounded to the nearest integer.
[b] A-STRs = autosomal STRs (number of sperm cells yielding profiles with ≥ 8 alleles in brackets).
[c] Numbers refer to haploid allele recoveries.
[d] only one of the two AMELY-positive sperm cells yielded a Y-STR profile.

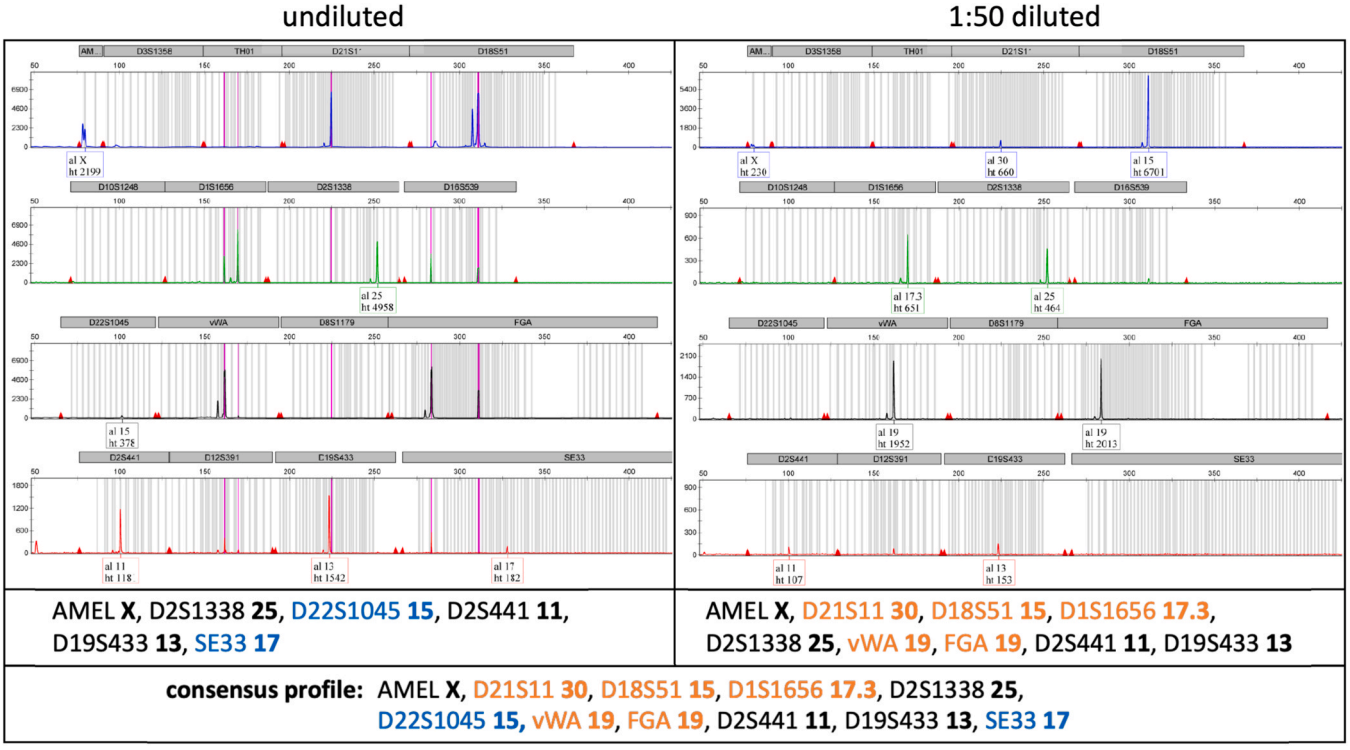

Fig. 3. Consensus autosomal STR profile from a single sperm cell isolated from a mock swab. Left panel: Electropherogram of undiluted PCR sample. Right panel: Electropherogram of the 1:50 diluted PCR product. The called alleles are noted below each panel, and the derived consensus profile is shown at the bottom. Blue letters: Alleles uniquely identified in the left panel; orange letters: Alleles uniquely identified in the right panel; black letters: Alleles identified in both panels. Genomic DNA was amplified with REPLI-g based WGA followed by STR analysis using PowerPlex ESX17. Positions of potential bleed-through signals are indicated by purple lines.

they could be duly micromanipulated ready for the WGA protocol. Although the micromanipulation steps would need to be thoroughly witnessed for corroborative purposes, such an approach would effectively lead to a combining of the casework cytology and DNA profiling steps. Furthermore, if historical samples such as slide preparations that contained sperm cells were available in a cold case review, we suggest that it might be feasible to adapt this method to recover and similarly manipulate such cells. A great advantage of the methods described here is that they do not require overly specialized equipment and subject to local validation would be relatively easily included into most forensic laboratory contexts. A disadvantage would be that, unlike LCM, the manual micromanipulation is only suited for low throughput analyses, requiring training and skill on part of the operator. Moreover, the application of WGA currently requires evaluation of electropherograms from both a diluted and an undiluted PCR sample in order to compile a consensus profile. Nevertheless, the method may serve as a supplementary tool for cases that would benefit from single cell analysis.

The micromanipulation method using adhesive-coated tungsten needle tips has been described before for larger bioparticles and requires an appropriate dilution of cells to avoid the unintended picking of more than one cell. In our hands, in all cases single sperm cells were isolated as revealed by the haploid genotypes of the analyzed STR loci as well as the sole presence of either AMELX or AMELY. An exception was locus D3S1358 of sperm 2 of donor B where both alleles of the donor were detected whereas the other loci displayed only one allele. This might be attributable to meiotic nondisjunction that has been described to occur

in sperm cells of healthy volunteers at a rate of 0.2% for chromosome 3 [32]. Alternatively, an ADI or a contamination cannot be ruled out.

The tungsten needle approach faced the difficulty of choosing a suitable substratum for dispersing the cells. The cells should not stick too strongly, as they otherwise become disintegrated during picking, and they should remain in place while microscopically assessed. In addition, the substratum should be translucent to allow microscopic monitoring. We found agarose gels to fulfill these requirements, and in addition these gels are easy to set up. Neither the adhesive tape nor eventually co-transferred agarose inhibited subsequent analysis.

Difficulties may arise when staining methods for sperm identification are used, and here it may be advisable to carry those out in suspension and to spread the cells thereafter onto the agarose plate. We have not used such techniques and, using high resolution optics, nevertheless allowed us to reliably identify sperm cells by morphology, as indicated by the haploid STR profiles. Identification of sperm cells was successful even if the cells had lost their tails, as was the case in the mock swabs.

In differential lysis protocols, sperm lysis by detergent requires DTT. In the first paper describing the application of Repli-g WGA on single sperm cells, Jiang et al. used a separate alkaline lysis buffer containing DTT to extract DNA from sperm cells and showed that incubation at an elevated temperature of 65 °C significantly improved lysis [33]. The current Repli-g single cell kit has incorporated these modifications, and we achieved lysis of single sperm cells by strictly following the commercial protocol using kit reagents.

The principal suitability of the Repli-g WGA for STR analysis had already been demonstrated by Jiang et al. who successfully genotyped five non-forensic STR loci (located on three different chromosomes) from single sperm cells that had been deposited into single tubes by limiting dilution of a sperm suspension [33]. For forensic purposes such an approach of mixture deconvolution is hardly suitable, because it requires the precise counting of relevant cell types and thus, cannot prevent co-isolation of contaminant cell types. Moreover, dilution follows the Poisson distribution, and thus a significant fraction of reactions will contain no cell or several cells, making analysis costly and inefficient. Recently, the successful application of Repli-g single cell WGA kit to STR analysis of diploid single cells was shown. In that study, cells of human lymphoblastoid cell lines were micromanipulated using aspiration capillaries and analyzed for autosomal and Y-STRs using CE-based or next generation sequencing (NGS)-based analysis using forensic STR kits [34]. In CE-based analysis, there were only few ADOs in the single cells analyzed, however allele balance was impaired and variable between the samples. Therefore, Repli-g WGA might be problematic if bioparticles are analyzed that may contain admixed DNA from a second individual. For single cell analysis, however, the Repli-g single cell WGA kit seems suitable.

Moreover, when analyzing haploid genotypes the problem of allele balance is of little relevance. Indeed we observed considerable allele imbalances with in part very high RFU values of the peaks that in turn caused high bleed-through peaks and occasionally high stutters, impeding interpretation at first. Our results show that interpretation of the electropherograms is nevertheless possible by in addition subjecting a 1:50 dilution of the PCR products to capillary electrophoresis and establishing the compound profile. The undiluted sample can reveal peaks that may drop out at the 1:50 dilution whereas in the latter bleed-through and high stutters will be removed. Based on our limited set of data, the gonosomal STR profiles generally seemed to display a better inter-locus balance than the autosomal STR profiles, since for the former a single dilution of WGA products often was sufficient to obtain most alleles. This observation would suggest that MDA-based WGA of loci on the same chromosome might result in more uniform amplification than of loci that reside on different chromosomes.

Effective MDA-based WGA requires long intact stretches of DNA which typically is not provided in forensic casework samples due to environmental exposure or aging of DNA traces. Protocols have been modified by circularizing the fragmented DNA to enable MDA to amplify DNA of compromised samples, however in a forensic context mixed results were obtained, and there was no benefit over conventional STR analysis [21,24,35]. DNA extracted from sperm cells, however, is known to yield high-molecular weight DNA [36], with little degradation even after several days post-coitum [37], and thus is expected to be suitable for MDA. This was corroborated by our successful STR typing of single sperm cells from the mock samples. Of note, from one of the mock swabs only one of two unknown contributors yielded STR profiles. Apart from statistically biased sampling, this may indicate an underrepresentation of sperm numbers of the respective individual, which may be due to an individually lower sperm count as is known from cases of infertility [38]. Furthermore, DNA quality of sperm cells can be variable and has been related to fertility as well [39], and it is possible that DNA from sperm cells of the respective individual might exhibit an increased number of strand breaks, thus interfering with successful MDA.

The autosomal STR profiles of the single sperm cells did not display pronounced stuttering as is typically seen after PCR-based WGA [19]. This may be related to the isothermal amplification process at 30 °C and the high processivity of the Phi29 polymerase [40] which renders replication slippage less likely. For this reason we assume that the possible slippage event observed in one of the sperm cells of the mock swabs was rather due to a replication slippage mutation in the germline than due to replication slippage during WGA (where in addition the original allele would have dropped out). Since Phi29 polymerase has a low error rate, our micromanipulation and WGA protocol may also be compatible with NGS-based STR analysis as suggested by the study of Chen et al. cited above [34].

Based on theoretical considerations, at least nine single sperm cells are required to be able to compile a diploid donor profile with a confidence of 95% [41]. As an efficient way to assign sperm cells of a mixture to their respective donors, the usage of Y-STR profiles has been suggested [14]. Because in the male germline the X chromosome does not undergo recombination, X-STR haplotypes can be used for the same purpose. We thus applied both Y- and X-STR haplotyping to reconstruct the diploid STR profiles of two donors by analyzing 11 or 7 single sperm cells, respectively. Of note, from the second donor, where we analyzed only 7 single sperm cells, the second allele of one STR locus was not detected which might be due to stochastic sampling. The reconstruction of diploid profiles will increase the statistical power of the DNA evidence in multiple-perpetrator rape cases by avoiding mixed profile statistical considerations. Moreover, diploid profiles will facilitate the identification of unknown suspects by DNA database searches.

5. Conclusion

We have established a simple method for the analysis of single sperm cells from sexual assault swabs. This method is based on a cost-effective micromanipulation technique and applies a commercial whole genome amplification kit, followed by conventional STR analysis. In terms of equipment and required reagents, the method could readily be implemented in forensic laboratories. However its application in casework analysis would require additional evaluation and laboratory-specific optimization of the micromanipulation and analytical steps. In fact, the WGA method might be combined with any technique for isolating sperm cells from casework samples. Our study shows that WGA is a viable option in forensics if applied to single sperm cells and may help resolving sexual assault cases in which standard analyses are not considered promising. Applications might include trace material where only few sperm cells are present or where multiple perpetrators were involved, as well as cold case reviews or any cases where low numbers of sperm cells have been identified on a slide but no DNA analysis was successful. Furthermore, the ability to assign single sperm cells to individual donors and to develop diploid consensus profiles from several single sperm cells may contribute to the clarification of multiple-perpetrator rape cases.

Funding

This work was funded by the German Federal Ministry of Education and Research (project no. 03FH019PX4). Further support was provided by a PhD scholarship to GMGT from the Institute of Safety and Security Research (ISF) of Bonn-Rhein-Sieg University of Applied Sciences.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Klaus Olek for his contributions to the project idea and his generous support during the initial phase of the project. We are also grateful to Hans Weiher who provided laboratory space and unconditional access to the micromanipulator-equipped microscope. Finally, we would like to thank the anonymous semen and buccal swab donors, and in particular the SPA Forensic Services for preparing the mock swabs. The project was funded by the German Federal Ministry of Education and Research (project no. 03FH019PX4). Further support was provided by a PhD scholarship to GMGT from the Institute of Safety and Security Research (ISF) of Bonn-Rhein-Sieg University of Applied Sciences.

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