International Journal of Legal Medicine
https://doi.org/10.1007/s00414-024-03285-1

REVIEW

Advancements in differentiation between sperm cells and epithelial cells for efficient forensic DNA analysis in sexual assault cases

Hirak Ranjan Dash[1]

Received: 11 April 2024 / Accepted: 30 June 2024

Abstract

Most of the sexual assault casework samples are of mixed sources. Forensic DNA laboratories are always in the requirement of a precise technique for the efficient separation of sperm and non-sperm DNA from mixed samples. Since the introduction of the differential extraction technique in 1985, it has seen significant advancements in the form of either chemicals used or modification of incubation times. Several automated and semi-automated techniques have also adopted the fundamentals of conventional differential extraction techniques. However, lengthy incubation, several manual steps, and carryover over non-sperm material in sperm fraction are some of the major limitations of this technique. Advanced cell separation techniques have shown huge promise in separating sperm cells from a mixture based on their size, shape, composition, and membrane structure and antigens present on sperm membranes. Such advanced techniques such as DEParray, ADE, FACS, LCM, HOT and their respective pros and cons have been discussed in this article. As current-day forensic techniques should be as per the line of Olympic slogan i.e., faster, higher, stronger, the advanced cell separation techniques show a huge potential to be implemented in the casework samples.

Keywords Differential extraction · Sperm cell · Sexual assault cases · FACS · LCM · DEPArray

Introduction

DNA profiling is the most commonly used technique in investigating sexual assault cases. The analysis of biological evidence and its potential use to provide leads toward a possible perpetrator is an important tool in handling sexual assault cases. Despite several advantages, the investigation of sexual assault cases is a major challenge in most forensic DNA laboratories across the globe. The success rate of a forensic DNA analysis in sexual assault cases depends on various factors such as the number of assailants, the quality of semen of the assailant, the time gap between the incidence and sample collection, and the sampling and storage conditions [86]. Besides, various technical and/or experimental variations also act as bottlenecks in forensic DNA analysis in sexual assault cases.

Most of the biological samples associated with the investigation of sexual assault cases originate from the victims' bodies. Hence, it is challenging for a medical examiner to collect a higher fraction of male cells in comparison to the female cells. The situation worsens when the time lapse between the incidence and sampling increases. Studies have shown that, ejaculation with a normal sperm quality of any man, the spermatozoa are detected in intimate swabs optimally up to 48 h and occasionally up to 6 days [24]. Detection of spermatozoa without a valid DNA test does not attribute to the possible origin of a perpetrator. For a successful DNA analysis, an optimum male-to-female DNA ratio (M: F) is also required. Besides a minimum template DNA requirement, the new generation autosomal STR kits also have a limitation when the male: female ratio exceeds 1:8 for Globalfiler (ThermoScientific) [55] and 1:19 for Fusion 6C (Promega Corp.) [21]. As most of the sexual assault samples tend to generate a mixed DNA profile, a sample having low M: F DNA might generate an uninterpretable DNA result.

The routine forensic DNA analysis involves extraction of DNA, quantification, amplification of targeted STR markers, genotyping, and interpretation of results. Most of the biological samples obtained in a sexual assault case are a mixture of female cells as well as male cells [4]. Once the

✉ Hirak Ranjan Dash
hirakdash@gmail.com; ranjan.dash_dc@nfsu.ac.in

[1] National Forensic Sciences University, Delhi Campus, Sector-3, 110085 Rohini, New Delhi, India

Published online: 12 July 2024

DNA is extracted from such samples, its quantity and quality determines the possibility of obtaining an interpretable DNA profile. Though Y-STR analysis has been deemed to be useful in such cases where a low M: F ratio is expected, it can only provide the lineage information of the perpetrator. Y-STR analysis does not lead to the individualization of the assailant (Court, [19]). Though recent studies have reported the usefulness of rapidly mutating (RM) Y-STRs [62] to differentiate between patrilineal relatives, such markers are not routinely used in most of the forensic DNA laboratories. Besides, in the absence of any mutational events during meiosis, RM Y-STRs cannot distinguish between the patrilineal relatives. Hence, most of the research has been carried out to separate the sperms from the non-sperm material to generate an interpretable DNA profile. As rightly described by Butler [12], forensic DNA protocols should be expected to be in a similar line to the Olympic motto i.e., faster, higher, and stronger. In this regard, the present article describes the currently available techniques to differentiate between the sperm and non-sperm fractions from a forensic biological sample obtained in a sexual assault case having a rapid, sensitive, and stronger investigative potential.

Differential extraction techniques

The use of chemicals to differentially lyse the sperm cells and non-sperm cells from a mixture of cells as observed in the sexual assault cases was first developed by Gill et al. [27]. As it was difficult to obtain bands from the sperm cells in a mixture sample, only female cells were lysed by a preliminary treatment using an SDS/proteinase K mixture. Sperm nuclei are invulnerable to such treatment due to the presence of cross-linked thiol-rich proteins in the cell membrane. During spermatogenesis, in the epididymal maturation stage, cross-linking of inter- and intra-protamines occurs by the formation of disulfide bonds present in thiol groups of protamines [13]. It causes stability and resistance of the sperm nuclear membrane. Further, the sperm material is lysed with SDS/proteinase K/ DTT mixture. The detailed workflow of this differential extraction technique is described in Fig. 1. Though most forensic DNA laboratories are still using this technique for the extraction of DNA from sexual assault biological samples, several modifications have been carried out in this protocol. In a modified two-step differential extraction method, Yoshida et al. [94] dried the vaginal fluid stains mixed with semen at room temperature overnight. In the first step, the mixed stain sample was lysed with TNE buffer (10 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl), 1% SDS, and 100 µg/ml proteinase K. In the second step, the sperm heads were lysed with lysis solution 2 (TNE buffer, 1% SDS and 100 µg/ml proteinase K, and 0.04 M DTT). Though the chemicals used in this method were similar to the protocol described by Gill et al. [27], the first step digestion temperature was maintained at 70°C which allowed the inhibition of DNase activity and the chance of sperm DNA to remain relatively intact.

Though DTT is widely used to lyse sperm heads differentially, several studies have reported the detrimental effect of DTT in the extraction of nucleic acid from sperm cells. As reported by Roszkowski and Mansuy [64], supplementation of DTT or β-mercapto ethanol is not sufficient enough to lyse the mouse sperm cells. As per this study, trizol supplemented with tris(2-carboxyethyl)phosphine (TCEP) allows complete, efficient, and rapid lysis of sperm cells. By the use of TCEP and bead-based homogenization, the DNA extraction technique has also become rapid and lysis of disulfide bonds has been facilitated without the use of proteinase K [89]. Thus, this technique can be deemed as an alternative to the conventional proteinase-K/DTT lysis of sperm cells for suitable use in forensic casework samples.

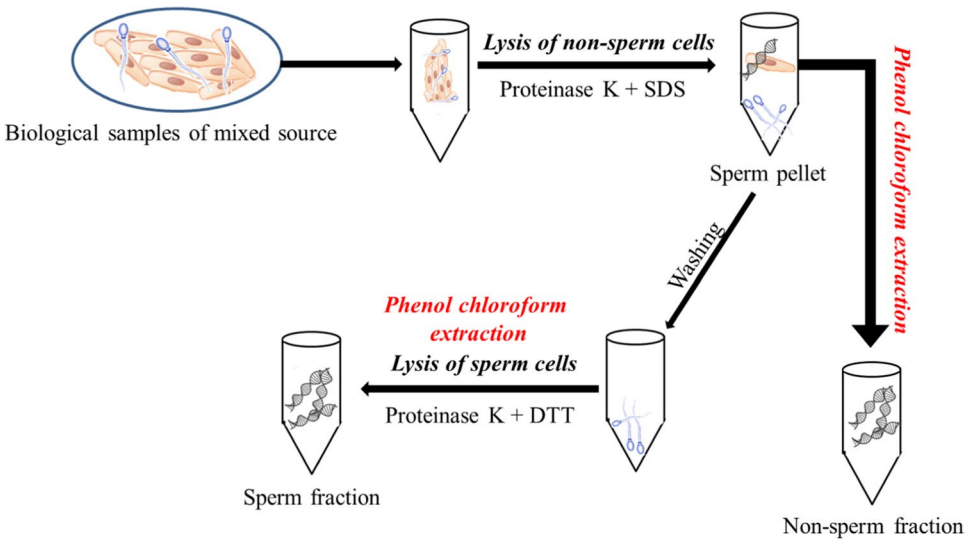

Fig. 1 Workflow of the conventional differential extraction technique

Over the years, much advancement has also been seen in the use of alternative buffers and incubation strategies for differential extraction of sperm DNA and non-sperm DNA. Using the conventional proteinase-K/DTT, increasing the pH of the buffer to 8.5 and incubating the cells at 42°C for 30 min has shown a 200–300% increase in the recovery of sperm cells from aged samples [54]. Similarly, an alkaline-based differential lysis technique has also been developed. Efficient lysis of sperm cells has been reported by using 1 N NaOH and the lysis of non-sperm material was achieved by using 0.1N NaOH. This alkaline method of lysis is considered to be advantageous to the conventional DTT method, as < 50% of male DNA present on a swab is captured by a standard extraction method where only DTT is used [40].

Despite several advancements in differential extraction strategies, the carryover of sperm DNA into the non-sperm fraction (NSF) is a major problem [31, 32]. If the sperm cells are co-lysed with the non-sperm cells, the sperm DNA may be lost in the NSF and it may also create a hindrance in profile analysis as the NSF can result in generating a mixed profile after genotyping. The success rate of a differential extraction strategy can be evaluated by using either the qRT-PCR technique or by analyzing a DNA profile. The percentage carryover of sperm DNA in the NSF can be calculated by using either Eq. 1 or Eq. 2 as provided by Hennekens et al. [36] as given below. Thus, any modification in the differential extraction strategy should be assessed in the laboratory before its implementation in the casework samples.

$$\% \ male\ contribution = \frac{\left[DNA_Y\right]_{NSF} - \left[DNA_Y\right]_{Free}}{\left[DNA_{Human}\right]_{NSF} - \left[DNA_{Human}\right]_{Free}} \quad (1)$$

$$\% \ male\ contribution = \frac{\left[RFU_{maleallele}\right]_{NSF} - \left[RFU_{maleallele}\right]_{Free}}{\left[RFU_{Total}\right]_{NSF} - \left[RFU_{maleallele}\right]_{Free}} \quad (2)$$

Automated and semi-automated differential extraction techniques

The standard differential extraction techniques conventionally used are both labor-intensive and time-consuming when performed manually. Most of these techniques involve several steps such as lysis, centrifugation, transfer of liquid, and washing. To minimize the human intervention in the differential extraction process, several automated and semi-automated techniques have also been developed.

Hamilton AutoLys STAR system

A recent study [76] described the use of Hamilton AutoLys STAR liquid handling assay-ready workstation for rapid and automated extraction of sperm DNA from a mixture sample. This technique uses the established chemicals and reagents in a standard differential extraction process. The only manual step involved in this process is to load the mixed samples in a proprietary AutoLys-A tube. After which, the incubation, centrifugation, sample transfer, wash, and separation steps are taken care of by the "hands-free" robotic system. The study on simulated sexual assault samples showed the advantage of this automated technique over the conventional manual technique. In this study, a higher amount of male DNA was yielded in samples with extremely low semen levels (< ~ 0.1 µl). Other advantages of this system include no cross-transfer of samples and high throughput, enabling the processing of 24 samples at a time.

Automated Differex™ system

The Differex™ system explores the commonly used differential extraction technique and automates every step of differential extraction. This system is used in combination with the DNA IQ™ system (Promega Corp.) and Slicprep™ 96 device. It has the capability of processing 48 samples for differential extraction within 5 hours including the incubation time. Certain protocol modifications such as additional proteinase K and DTT, having a longer incubation time, and using additional steps when removing the solid support from the digestion solution showed a comparable result with the established manual chelex1-100-based method [81]. Another study compared the use of the Differex™ system along with the DNAIQ™ system, QIAamp® DNA micro kit, phenol/chloroform extraction, and the conventional two-step method. When the peak height of all the techniques were compared, the highest peak height was observed with the Differex system + QIAamp DNA micro kit combination, whereas, the lowest peak height was observed with the Differex system + phenol/chloroform extraction combination [79]. It shows that the faster Differex system has equal efficiency with the two-step method of differential extraction. Another modification by Ng et al. [59] resulted in a sensitive, high sperm DNA recovery, robust technique when Maxwell-16 DNA extraction system (Promega Corp.) was incorporated as a semi-autonomous method. Additionally, it significantly reduced the processing time of sexual assault samples.

Sampletype I-sep DL

Sampletype I-sep DLMB extraction system (Biotype) has been evaluated to separate sperm DNA from non-sperm DNA using the Prepfiler forensic DNA extraction kit (Thermo Scientific). I-sep DL is a combination of lysis buffer DL-MB and i-sep® DL spin basket. Buffer DL-MB lyses non-sperm cells with the help of proteinase K while sperm cells remain intact. I-sep® DL spin basket seals

the fluid barrier reversibly and retains intact sperm heads above the fluid barrier (Bogas et al. [11]). I-sep differential method was found to be more sensitive than the pellet differential method as it was able to extract quantifiable male DNA from as low as 0.00005 µl of semen input. In the same study White et al. ([87]) reported that, in comparison to the 15–88% recovery efficiency of sperm fraction DNA of pellet differential method, the i-sep® differential method could able to recover > 99% in the initial extraction. Thus, the i-sep® method provides a robust, automated, and sensitive method of differential extraction strategy for its useful application in sexual assault casework samples.

QIAcube HID differential washing station and BioRobot EZ1

QIAcube HID differential washing station (QIAGEN) is an automated platform delivering differential separation and wash protocol for sexual assault samples. It enables differential fraction separation and sperm lysis from 6 samples within 1 hour. This is the only dedicated automated differential washing station to the best of our knowledge. When its efficiency was compared with six other protocols such as Differex™, Sampletype i-sep®DL, Sampletype i-sep®SQ, GEN-IAL® First-DNA all tissue kit, the Erase sperm isolation kit, it outperformed all other techniques in terms of male DNA recovery and male to female DNA ratio except Sampletype i-sep® SQ method [70]. Another study in a similar line showed that the automated QIAcube cell separation and lysis method is capable of differentially lysing and separating sperm cells from mixed cells and this technique is more effective in increasing the total human: male DNA ratio in comparison to the equivalent manual methods [28]. Thus, this automated instrument can be used for efficient use in the differential extraction of sperm DNA in sexual assault casework samples.

DNase digestion-based (Erase sperm) system

Erase sperm isolation kit (PTC laboratories, Columbia, MO, USA) is based on the routinely used differential extraction method. In the first lysis step, all non-spermatozoa cells are lysed with mild reagents. Subsequently, a centrifugation is carried out to pellet down the sperm cells. As a modification to the conventional process, the pellet containing spermatozoa is treated with deoxyribonuclease which digests the free DNA of lysed non-spermatozoa cells. This step ensures that only sperm DNA is found in the sperm fraction after the second lysis step using DTT [33]. In comparison to the other available differential extraction methods, the Erase method using DNase is more efficient for removing non-sperm DNA facilitating the generation of male profiles from samples containing a large amount of female DNA [45]. A significant increase in the RFU (1021) was observed in the sperm fraction samples extracted using the DNase system in comparison to the manual differential extraction technique (RFU: 678) [25]. Such a result was also corroborated in five crime laboratories located in the USA, Switzerland, and Germany that participated in the study. This suggests that the DNase digestion-based system is a better alternative to the conventional differential extraction systems in terms of obtaining STR profiles of male DNA fractions.

On chip cell lysis methods

To expedite the extraction of sperm DNA from the mixed cell suspensions as obtained in sexual assault cases, different microfluidic chips have been described for simultaneous sperm capture and lysis. Bienvenue et al. [10] fabricated a microdevice of Borofloat glass and explored the microchip-solid phase extraction (SPE) strategy. The lysis buffer was standardized to contain 6 M GuHCl solution and 40 mM DTT. Though this microchip was standardized for extraction of neat semen samples, this technique provided a huge potential for one-step lysis of sperm cells as evidenced by the obtained electropherograms. Inci et al. [41] developed another microchip containing a unique oligosaccharide sequence (Sialyl-LewisX), which selectively captured the sperm cells and subsequently lysed them with tris(2-carboxyethyl)phosphine (TCEP) in Triton X-100 followed by processing through proteinase K and spin column. Different workers have subsequently developed microfluidic devices for selectively capturing sperm cells from a mixture of cells [37, 39, 65]. Vasilescu et al. [85] fabricated a 3D printed inertial microfluidic device which was found useful in recovering > 96% of sperm cells from a mixture of sperm cells, RBCs, WBCs, ECs and cancer cells in less than 5 min. Another microfluidic device was developed to separate the sperm cells by exploring their rheotaxis behavior. Testing this device in a clinical setup has shown separation efficiency of 100% for the viable and highly motile sperms [67]. Though most of the advancements have been validated for the clinical practice and have been found in the field of assisted reproductive technology (ART), such devices are also deemed useful in forensic setup.

Sperm cell separation techniques

Over the last few decades, several attempts have been made to separate sperm cells from the non-sperm cells. Most of these techniques are based on immunological and affinity techniques, laser capture microdissection technique, DEPArray™ systems, fluorescence-activated cell sorting, acoustic trapping, pressure-cycling, on-chip method, and holographic optical trapping (HOT).

Filtration techniques

Chen et al. [15] demonstrated the use of an 8-µm nylon mesh membrane filter for selective trapping of larger non-sperm cells and passing through of small-sized sperm cells. Epithelial cells are about 50µm in diameter whereas, spermatozoa are 50µm in length but < 6µm in width [9]. In another study, Ladd et al. [47] reported a track-etched filter or a laser track-etched filter, or a combination of the two for separation of sperm cells and non-sperm cells. These techniques showed promising results using the pristine sperm cells whereas fail to achieve the greater goal while dealing with the casework samples. In this regard, in a recent study, Sinha et al. [73] validated a novel method 'SpermX™' for selective trapping of sperm cells on the SpermX™ membrane. It uses a nanotechnology-based polymer membrane which acts as a separation medium for the selective trapping of sperm cells. The SpermX method yielded a 3–6 fold increase in the recovery of sperm DNA in comparison to the traditional differential extraction techniques. Inter-laboratory validation of this technique showed promising results in sexual assault casework samples by generating seven-fold increases in the unshared male alleles in comparison to the conventional SE methods [71]. Thus, the SpermX technique can be deemed to be more useful among the available filtration techniques for efficient use in sexual assault cases due to its high competency as well as capability of automation.

Hydrodynamic sorting

The use of hydrodynamic effect has been used for cell separation for decades. Several microdevices have been developed for cell separation using inertial force at a high flow rate [74]. The membrane of non-sperm cells is weak in comparison to the sperm cells having low Reynolds numbers (Re < 20). Reynolds number is the ratio of inertial forces to the viscous forces. It is used to determine whether a fluid is in laminar or turbulent flow. It is recommended that, a Reynolds number ≤ 2100 indicates laminar flow. Hence, maintaining a high flow rate may lyse the non-sperm cell membrane due to the turbulent flow. The principle of the hydrodynamic sorting-based cell separation is depicted in Fig. 2. In this regard, Yamada et al. [93] proposed a 'pinched flow fractionation' technique for size-based separation of cells using the pinched-expanded channel with a bifurcated inlet. Such techniques are useful for cells having a regular shape. However, as the shapes of non-sperm cells vary greatly, the pinched flow fractionation technique or hydrodynamic filtration technique is of limited use to separate non-sperm cells in sexual assault casework samples. Liu et al. [53] fabricated a microfluidic device based on the hydrodynamic effect of flow at low Reynolds number which showed promising results as 94.0% of pure sperm fraction DNA could be obtained from mixed samples. Berendsen et al. [9] also developed a microfluidic chip that separates spermatozoa from erythrocytes by the tumbling behavior of spermatozoa in pinched flow fractionation. Though this technique has been standardized for efficient separation of freshly prepared sperm cells (95% separation) from erythrocytes, this technique can also be explored for its forensic use in sexual assault cases.

Acoustic differential extraction (ADE)

One of the most promising approaches for the separation of sperm cells and non-sperm cells is acoustic manipulation of cells, i.e., the use of sound waves to move the cells. Most of these microfluidic devices employ a piezoelectric transducer (PZT) which vibrates and allows the formation of a standing acoustic wave and cells are separated based on their size, shape, density, and compressibility [48]. Clark et al. [16] fabricated a microfluidic chip composed of glass, polydimethylsiloxane (PDMS), and poly(methyl methacrylate)

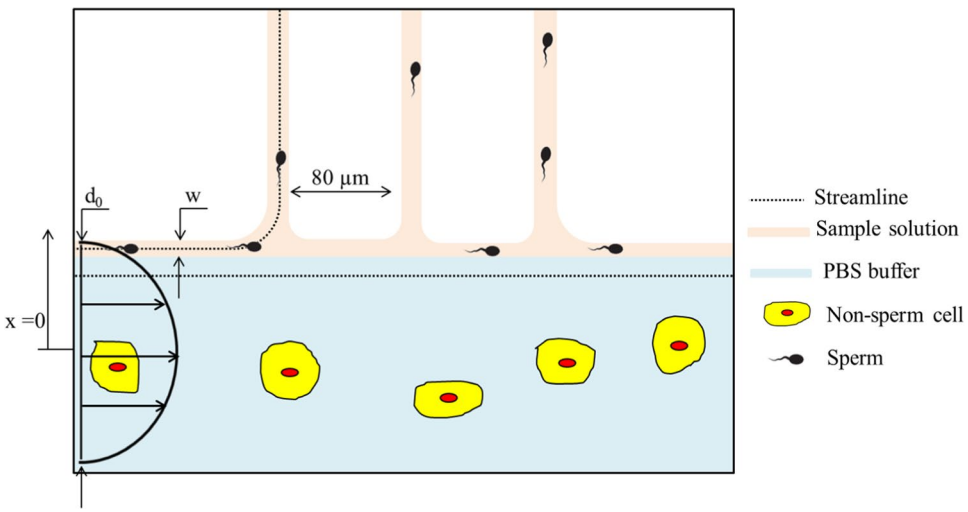

Fig. 2 Principle of hydrodynamic sorting based separation of sperm and non-sperm cell highlighting the recovery of sperms in parallel capillary tubes (modified from Samuel et al. [65])

(PMMA) layers. In this system, the required system operation time is 7 min per sample and a sperm pellet is collected in the final volume of 30µl. Reduction of the processing time to as low as 7 min/ sample and the high rate of male and female DNA (1:40) separation are two major advantages of this system. Norris et al. [60] described an acoustic differential extraction (ADE) technique utilizing the size difference between sperm cells and free DNA which retains the sperm cells. To accommodate a standing wave in the ADE device, the depth of the microchannels and the thickness of the reflector layer should be in tandem with the transducer's operating frequency. Hence, this microchannel accommodated a single λ/2 or 3λ/2 standing wave and the transducer was operated at 11.6 MHz, where, λ is the wavelength of the optimal transducer resonance frequency. Purified sperm and non-sperm materials were separated from the sexual assault samples in 14 min using this device. In the samples prepared with 12µl of semen, an increased recovery of male DNA as 87 ± 24 pg/ µl was achieved, which showed the purity of the sperm DNA. Another study demonstrated a microchip having glass–PDMS–glass (GPG) resonator to isolate sperm cells from large volume samples. This system showed its effectiveness in processing samples with volumes up to 300µl and cell concentrations as low as ~ 10 cells/µl [90]. Acoustic capture is more suited for the sperm cells due to their relatively uniform morphology and non-variable size in humans [8]. This enables the tuning of the microfluidic trapping sites to customize a precise size. Besides, the application of high-frequency vibrations (7–9 MHz) and adjustment of the applied frequency of each chip, multiple vertical trap zones can also be created which increases the chance of simultaneous capture of a high number of sperm cells. Thus, acoustic-based differential extraction techniques are considered to be highly effective in terms of purity of sperm DNA and high throughput.

Dielectrophoresis (DEP) array

Dielectrophoresis (DEP) is the movement of dielectric particles in a heterogeneous electric field. A single-use microfluidic system containing an array of electrodes is used. The array has di-electrophoretic (DEP) cages that capture and manipulate single cells. Negative electrophoresis facilitates cell capture by creating an electric field above a subset of electrodes which is in counter phase with the electric field of adjacent electrodes. Further, the cell is moved to a recovery chamber by changing the electric field pattern. Though this technique was initially developed for the detection of circulating tumor cells, nowadays, the DEParray technique is also used to categorically select sperm cells from a mixture of cells.

The difference in cellular size, chemical composition, and membrane structure allows the sperm and non-sperm cells to behave differently when they are placed in a non-uniform electric field. Williamson et al. [88] demonstrated the DEParray workflow and it significantly increased the chance of obtaining a single source profile from 32.1% (for differentially extracted samples) to 96.2% (for DEPArray™ processed samples). The left-right protocol was developed by Silicon biosystems which separates sperm and non-sperm cells by using DEP cages where sperm cells move to the right-hand side and non-sperm cells move towards the left-hand side of a DEPSlide™ chip. A recent study measured the DEP force of human sperm to be 3 pN and the WBC as 42pN. This result adds a new approach to the validation of DEParrays™ for their capable use including sperm cells [44].

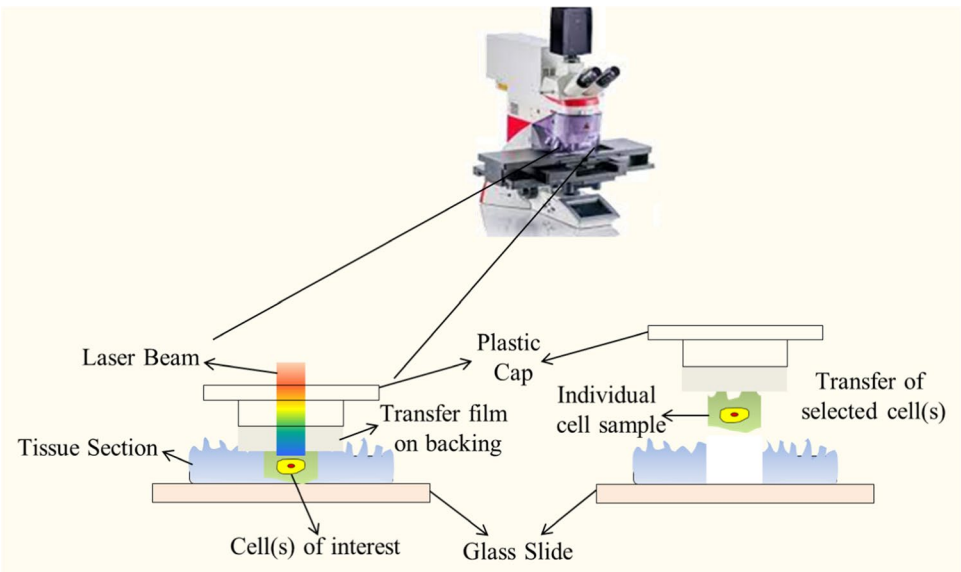

Fig. 3 Generalized process of cell picking by laser-capture microdissection (LCM) system (modified from Simone et al. [72])

Flow cytometry

Fluorescence-activated cell sorting (FACS) relies on differential morphology, ploidy, cytokeratin content, and/or surface phenotype for the separation of cells. Though a limited study is available on Flow cytometry-based separation of sperm and non-sperm cells from forensic samples [23, 69], this technique has shown promising results for its application in casework samples. Sperm Hy-Liter™ staining kit (Independent Forensics) in conjugation with FACS has successfully separated sperm and non-sperm cells of 1: 500 ratios [23]. Vaginal epithelial cells contain cytokeratin which is specific for epithelial-derived cells and is absent in spermatocytes. When a mixture of spermatozoa and vaginal epithelial cells are stained with monoclonal antibodies (mAb) conjugated with fluoroisothyocyanate (FITC), all vaginal cells show a distinct green fluorescence on flow cytometry due to the presence of distinctive cytokeratin, whereas, sperms are not stained as green. Thus, FACS separates the stained and unstained cells [69]. Schoell et al. [68] differentially labeled the sperm cells with CD45-FITC and were able to sort 1 sperm cell to 10 vaginal cells, at a sensitivity of 92%. This showed a significant advantage over the routine preferential lysis method whose sensitivity was reported to be 56%. Though the FACS technique has shown promising results in separating sperm cells from a mixture, this technique should be validated in casework samples before its routine application in forensics. FACS technique has also shown promising applications in tissue differentiation based on fluorescence intensity expression of A, B, and H antigens in blood and saliva samples [7].

Despite of showing promising results in separating sperm and non-sperm materials, FACS technique has shown several potential drawbacks. FACS relies on the presence of certain molecules on (or in) the cells, which can be detected once bound with specific antibodies coupled to fluorescent markers. Though the ploidy of the sperm cells is a distinguishing feature from that of other non-sperm cells, FACS technique requires the use of DNA binding markers such as DAPI or Hoechst to differentiate cells based on ploidy. Similarly, morphological difference will impact the diffraction of light that may be detected and used for different cell types; however, FACS technique relies mainly on the cell differentiation using fluorescent labeled antibodies. Additionally,

Table 1 Sperm specific proteins useful for antibody based sorting of sperm cells from mixtures and their functions

Sl. No.	Sperm proteins	Function	Specificity	References
1	Intra acrosomal protein (SP10)	Plays an important role in egg-sperm binding	Specific to the testis	Hamatani et al. [34]
2	Motile sperm domain-containing protein 3 (MOSPD3)	Involved in sperm motility and involved in spermiogenesis	Mammalian specific sperm membrane protein	Li et al. [51]
3	Acrosin binding protein (ACRBP)	Regulates the autoconversion of proacrosin to intermediate forms of acrosin	Mammalian sperm	Kato et al. [43]
4	sperm adhesion molecule (SPAM1) (PH-20)	a glycosyl phosphatidy linositol-anchored sperm hyaluronidase enabling acrosome-intact sperm to reach the egg zona pellucida during fertilization	Sperm head specific	Zhao et al. [96]
5	A distintegrin and metalloprotease 2 (ADAM2)	Involves in the egg-sperm membrane fusion fertilization process	Testis and epididymis	
6	JNK-associated leucine zipper protein (JLP)/ SPAG9	A potential endogenous antifibrotic factor	Sperm associated antigen	
7	Human lipocalin 6 (hLCN6)	Binds to sperm and plays important role in sperm maturation	Epididymis-specific secretor protein	Chen et al. [14]
8	A-kinase anchoring protein 3 (AKAP3)	Essential for the formation of the specific subcellular structure of the sperm flagellum, motility of sperm and male fertility	Sperm and sperm flagellum	Xu et al. [91]
9	Beta-1,4-galactosyltransferase 1 (B4GAL-T1)	Plays a vital role in sperm-egg interaction	Sperm associated antigen	Ren et al. [63]
10	NUH2 (sperm carbohydrate antigen)	Responsible for the immobilization of human sperm	Sperm specific, expressed at low level in normal cells	Tsuji et al. [78]
11	HS21	NA	Found in sperm with intact Acrosomes	Zeginiadou et al. [95]
12	Glycosyl phosphatidylinositol-anchored hyaluronidase	Role in proper sperm–plasma membrane structure formation	Testicular germ cells	Andre et al. [3]

Table 2 Power of new generation autosomal multiplex PCR kits available in the market for their application in sexual assault mixed biological samples

Sl. No.	Multiplex kits	Manufacturer	Sensitivity	Complete mixed profile with M:F ratio	Reference
1	GlobalFiler®	Thermo Scientific	125 pg	1: 5	Ludeman et al. [55]
2	VeriFiler™ Plus		62.5 pg	1: 7	Green et al. [30]
3	Investigator® 24Plex QS	Qiagen	125 pg	1: 10	Kraemer et al. [46]
4	Investigator 26plex QS Kit		125 pg	1: 5	Marcińska et al. [56]
5	PowerPlex® Fusion 6C	Promega Corp.	250 pg	1: 2	Ensenberger et al. [21]
6	SureID® PanGlobal	Health Gene technology	125 pg	1: 5	Marcińska et al. [56]

FACS technique involves expensive instrument having very low through-put. High chance of contamination is another major problem of this technique as the whole fluidic line used to process the sample is the same between all samples. Though contamination issue can be minimized by the use of a disposable cartridge for cell sorting, it may result in a higher sorting price as well. Another problem associated with the FACS technique is that, it does not prevent cell-free DNA to be sorted along with the cells of interest when processing case work samples. In conditions where the non-sperm cells number is very high compared to the number of spermatozoa to sort, and the sample is somewhat degraded, a significant amount of non-sperm free DNA floating around the spermatozoa might be sorted along spermatozoa.

Laser capture microdissection/optical tweezers

The laser capture microdissection (LCM) technology combines the existing light microscopic instrument with laser beam technology and targets specific cells for their separation. Majorly there are two classes of LCM techniques, i.e. ultraviolet (UV) cutting systems and infrared (IR) capture systems. After the identification of target cells by microscopy, the cells are isolated by focused laser energy (UV systems) or transferred to a thermoplastic polymer with the formation of a polymer-cell composite (IRsystems). The generalized principle of the LCM technique is depicted in Fig. 3. The UV systems being completely non-contact systems are preferred in forensics to avoid the risk of contamination [83]. Elliott et al. [20] used an IR system to separate the spermatozoa from vaginal epithelial cells and reported the greatest chance of obtaining male genotypes in comparison to the differential lysis. In this study, 93.75% samples resulted in the generation of a greater likelihood ratio (LR) in the magnitude of 10^6 to 10^{10} compared to the conventional differential lysis method. When a UV system was evaluated for the microdissection of sperm cells, useful DNA profiles except for a few dropouts were observed from 10, 20, and 30 cells from a smear. Subsequently, a UV system in combination with membrane-coated slides has been developed. Though this allows a greater success rate in comparison to the UV or IR systems alone, this technique is expensive and was found to be more useful in fresh samples [66]. The membrane coated systems were improved further by the use of polyethylene-naphthalate (PEN) membrane slides and the sperm cells were stained with Hy-Liter™ staining before LCM. This modification allows the capture of > 100 sperm cells and the recovery of 100% alleles [6]. Further, the LCM in conjugation with low-volume PCR showed the generation of the complete profile from as low as 15 sperm cells with 80% reproducibility [49, 50]. In this regard, an automated scanning method has been developed by identifying sperm heads stained with sperm Hy-Liter™ and using the image processing AxioVision commander module (Carl Zeiss) followed by laser pressure catapulting using a pulsed nitrogen UV-A laser ($\lambda_{355\ nm}$). This robust method successfully recovered 30 spermatozoa from post-coital samples and successfully generated complete DNA profiles without any female alleles [84].

The optical tweezer technique is more similar to the LCM technique. However, cells remain fixed on the microscope slide in the LCM system, whereas, optical tweezers act in an aqueous system and operate without the need of staining, fixing, or melting/catapulting cells to another surface. Optical tweezers have several advantages as < 60 sperm cells are required to obtain a complete STR profile, whereas, most of the antibody-based methods discussed have successfully been tested on samples with ~ 10^5 sperm cells [92]. As antibodies are not required in optical tweezer techniques, the associated complications of antibody binding such as specificity and efficiency do not exist. A study has reported this technique to be more effective in separating sperm cells from a mixed sample. ≥50 sperm cells were tweezed using this method and complete or near complete STR profiles were consistently generated in mock sexual assault mixtures [5].

Antibody-based capture

Antibody-based capture is a potential method of separating sperm cells from the vaginal epithelial cells. Sperm

Table 3 Pros and cons and technology readiness level (TRL) of the available techniques for analysis of DNA from sperm cells in mixed samples

Technique	Advantages	Disadvantages	TRL (1 to 9 Scale)	Reference
SDS/Proteinase K/ DTT based differential lysis	• Cost effective • Selective lysis of Sperm cells	• Time consuming • Laborious • High carryover of non-sperm fraction	9	Vuichard et al. [86]
Automated differential lysis techniques	• Minimal human intervention • No cross-transfer of samples • High-throughput	• High cost • Requirement of additional infrastructure • Mostly closed systems	9	Ng et al. [59]
On chip cell lysis methods	• Minimal sample consumption • Can be automated	• Lengthy incubation to facilitate sperm capture	7	Bienvenue et al. [10]
Filtration techniques	• Selective trapping of sperm cells • Capable of automation • No limit on sample volume	• Less throughput	8	Sinha et al. [73]
Hydrodynamic sorting	• > 95% efficient in separating sperm and non-sperm cells • Capable of automation	• Variation in size of non-sperm cells • High flow rate may lyse the non-sperm cell membrane	5	Liu et al. [53]
Acoustic differential extraction	• Reduction in processing time • > 95% efficient in separating sperm and non-sperm cells • Accommodates a large range of sample volumes • Capable of automation	• Complex antibody preparation and handling • Failure to isolate sperm at concentrations below 10^3 cells/ ml.	8	Clark et al. [16]
Dielectrophoresis (DEP) array	• Single stain retention capability • Individual cell capture • Useful in trace evidences, less sperm cells	• Cell staining and lengthy incubation • Costly	7	Williamson et al. [88]
Fluorescence-activated cell sorting (FACS)	• High specificity • Highly sensitive	• Increasing risk of clogging when biological material is used • High cost of the instrument	7	Bakdash et al. [7]
Laser capture microdissection	• No contaminating female component • Useful for samples containing low number of sperm cells • Capable of semi-automation	• Huge expertise required to remove targeted cell(s) through laser manipulation • High cost of the instrument	8	Axler-DiPerte et al. [6]
Antibody-based capture	• Capable of automation • Diverse proteins found on sperm cell	• Useful for samples containing > 10^4 sperm cells/ml • Not suitable for old samples • Decaying of tail, flagellum, and middle piece of sperm affects the capture • Non-specific bindings may occur	7	Alsalafi and Goodwin, [1]
Holographic optical trapping (HOT)	• Scope of complete automation • Full profile from as low as 10 cells	• Requirement of skilled individuals to perform the experiment	7	Valle et al. [82]
MACSprep™ forensic separation	• Amplification ready samples within 2 h • High purity of sperm fractions • Already available with automation	• Lower recovery compared to ERASE for samples with low number of spermatozoa	8	Grosjean et al. [33]
Low volume PCR (LV-PCR)	• Useful in LT DNA • Useful in combination with FISH and LCM • Capable of automation	• Not 100% reproducible	8	Feng et al. [22]

Table 3 (continued)

Technique	Advantages	Disadvantages	TRL (1 to 9 Scale)	Reference
Sensitive multiplex kits	• High sensitivity • Robust • Inhibitor tolerance capacity • Capable of automation • Resolution of major and minor contributors • Commercially available kits	• High cost of the multiplex kits	9	Marcińska et al. [56]
Probabilistic genotyping systems	• High resolution of complex mixtures • Drop-in and drop-out factors taken into consideration • Peak height ratio taken into consideration	• Complex results to interpret • Requirements of laboratory specific large datasets • Unclear method of prediction	9	Gill et al. [26]

TRL 1: Basic principles observed and reported; *TRL 2*: Technology concept and/or application formulated, *TRL 3*: Analytical and experimental critical function and/or characteristic proof of concept, *TRL 4*: Component and/or breadboard validation in laboratory environment, *TRL 5*: Component and/or breadboard validation in relevant environment, *TRL 6*: System/subsystem model or prototype demonstration in a relevant environment, *TRL 7*: System prototype demonstration in an operational environment, *TRL 8*: Actual system completed and qualified through test and demonstration, *TRL 9*: Actual system has proven through successful mission operations.

cells are intrinsically unusual in many cellular and functional features and more than 100 different proteins have been described in human sperm cells using conventional approach. With the advent of the proteomic approach more than 1000 different proteins have been identified in sperm cells [2]. Though different proteins have been described in human sperm, proteins specific to the mature sperm cells are of high forensic interest. However, a study showed that, 3.2–3.6 million/ml immature sperm cells are present in semen [61]. Hence, proteins specific to both mature and immature sperm cells are of forensics interest which have been listed in Table 1.

Two anti-sperm antibodies i.e. SP17 polyclonal antibody and SP10 intra acrosomal protein antibody Hs-8 were evaluated by Alsalafi and Goodwin [1] and separation of spermatozoa was achieved with sperm concentration 10^3/ml to 10^4/ml. SP17 called as the sperm autoantigenic protein 17 or sperm protein 17 was initially characterized by its involvement in the binding of sperm to the *zona pellucida* of the oocyte. Recent studies indicate that it is also involved in additional cell-cell adhesion functions such as immune cell migration and metastasis [42, 80]. Magnetic beads-based separation of sperm cells using a monoclonal antibody against MOSPD3 (motile sperm domain-containing protein 3) was found to be effective for mixed samples containing 10^3/ml to 10^4/ml of sperm cells [51]. Another study found the magnetic bead conjugated acrosin binding protein (ACRBP) antibody to be more effective and a 90% genotype success rate was observed in > 1:100 ratio of sperm cells and female buccal epithelial cells [52]. Similarly, antibodies against PH-20, a sperm adhesion molecule (SPAM1) have also been found to be effective in selecting sperm cells. In clinical setup, 42.79–44.08% sperm cells were reported to be localized using SPAM1 [29]. Further, mixed samples having sperms of more than one individual could also be separated successfully based on anti-A, anti-B, or only anti-H antibodies followed by the LCM technique [75]. Though antibody-based testing has been established as a suitable technique for presumptive and confirmatory testing of spermatozoa and seminal fluid testing, such a technique can also be deemed to be suitable use in sperm separation from mixed samples as well.

Holographic optical trapping (HOT)

Holographic optical trapping (HOT) is an improved and advanced variant of optical trapping having the scope of complete automation. Optical trapping focuses laser beams of visible to near IR range to form traps that can trap and move particles of nm to μm sizes. Though non-holographic optical trapping techniques were used earlier, their inability to create large number of optical traps, and fewer throughputs has led to the discovery of HOT. HOT employs a hologram (phase mask) and shapes a laser beam's wave front by splitting a single beam into multiple optical traps which trap the sperm cells [77]. Mico et al. [57] produced the first holographic image of a spermatozoon. Though HOT has been widely used in visualizing the morphology of abnormal sperm from a pathological point of view, it has shown huge potential in forensic applications as well. A droplet-based optical trapping has also been reported to be useful in separating sperm cells in mock forensic samples. This method demonstrated the generation of full STR profiles from as low as 10 cells [82].

Table 4 Comparative account of the research progresses and their observations in processing sexual assault samples

Sl. No.	Technique used	Type of sample tested	Condition of the sample	Recovery rate/ purity	Reference
1	Differential Extraction using alternative buffer and incubation strategies	• Mock sexual assault sample • Swab from the cheeks + 0.5 µl of Semen having ~ 50,000 sperm cells/ µl	• Freshly prepared samples • Aged mock samples, stored up to one year	• 85% recovery of sperm cells from one week old samples and 68% recovery from one year old samples • Full STR profiles obtained from 500 – 100 cells • Peaks heights were found higher compared to conventional DE method	Lounsbury et al. [54]
2	Hamilton AutoLys STAR system	• Mock sexual assault samples prepared by adding 50 µL of diluted semen in PBS to cotton swabs containing vaginal or female buccal cells • Four authentic post-coital swabs (24, 48, 72, and 96 h post-coitus)	• Freshly prepared mock samples • Freshly used post-coital samples	*For samples having buccal swabs mixed with 0.02 µl of semen* • 1.5:1 M:F ratio observed in manual DE • 1.9:1 M:F ratio observed using STAR system *For post-coital samples* • 36 ng, 110 ng, 15 ng and 11 ng of Y observed in sperm fraction	Timken et al. [76]
3	Automated Differex™ system	• Mock sexual assault samples prepared by spiking buccal swabs of female volunteers with 100 µl of diluted semen • Previously-tested semen-negative intimate casework swabs from closed cases spiked with 100 µl of diluted semen	• Fresh samples air dried overnight	• 193 ng male DNA in sperm fraction isolated using Differex™ system compared to 148 ng male DNA isolated using manual DE (1:10 semen dilutions) • 15 ng male DNA in sperm fraction isolated using Differex™ system compared to 12 ng male DNA isolated using manual DE (1:100 semen dilutions)	Ng et al. [59]
4	Sampletype I-sep DL/ DNA IQ™ or Sampletype I-sep DL/ PrepFiler™	• Mock samples prepared by adding 10 µl of concentrated buccal cells with 50 µl of diluted semen • Mock post-coital samples prepared by high vaginal swab spiked with 0.005 µl neat Semen	• Freshly prepared mock samples	• 0.978 ng to 38.085 ng male DNA in sperm fraction isolated using Sampletype I-sep DL/ DNA IQ™ • 0.0017 ng to 0.6492 ng male DNA in sperm fraction isolated using Sampletype I-sep DL/ PrepFiler™ • All 40 markers were amplified above the analytical threshold	White et al. [87]
5	DNase digestion-based (Erase Sperm) system	• Mock samples prepared by adding 10 µl and 3 µl of 10% semen • Vaginal swab from volunteers after 5 minutes, 6 hours, 24 hours, 34 hours, 48 hours, or 58 hours after a single sex act • Samples were process in parallel in 5 laboratories • Three vaginal swab and one underwear processed in one laboratory	• Freshly prepared mock samples • Timed post-coital swabs	• Up to 72.0 ng of male DNA in Sperm fraction • Up to 193% of male DNA • Swab cuttings processed with Erase generated full male profiles at each time point (5 min, 6 h, 24h, 34h, 48 h or 58 h), while the profiles obtained with Differex™ were either mixtures or female profiles	Garvin et al. [25]

Table 4 (continued)

Sl. No.	Technique used	Type of sample tested	Condition of the sample	Recovery rate/ purity	Reference
6	QIAcube cell separation and lysis method	• Mock samples prepared by adding 3 µl of neat semen to female dried buccal swab sample	• Freshly prepared mock samples were evaluated	• > 75 ng of male DNA yielded in all samples Up to 72.0 ng of male DNA in sperm fraction • 1: 1.04 M:F ratio obtained • 1.5 median number of female STR alleles were detected in the mixture compared to 9 female STR alleles in manual organic extraction method	Goldstein et al. [28]
7	Microchip-solid phase extraction (SPE) strategy	• Simulated forensic samples were prepared by members of the Broward Sheriff's Office Crime Laboratory	• Freshly prepared mock samples were evaluated	• > 79 to 188 pg/µl male DNA yielded • Confirmed high DNA recovery with efficiency between ~ 52.8% and ~ 88.6%	Inci et al. [41]
8	3D printed inertial microfluidic device	• Mock samples prepared in two sets • *Set 1*: Solution of Sperm + RBCs + WBCs + Epithelial cells • *Set 2*: Sperm + K562 cells (chronic myeloid leukaemia cells)	• Freshly prepared mock samples were evaluated	• > 96% sperms were recovered • A single sperm spends < 0.25 s in the device	Vasilescu et al. [85]
9	Laser track-etched filter	• Mock samples prepared by adding 100,000 sperms to buccal swabs • 10 sexual assault evidentiary samples from nine previously adjudicated, non-probative cases	• Freshly prepared mock samples • Timed sexual assault evidentiary samples	• Only the mixture of 12,500 epithelial cells with the 100,000 sperm filtered effectively	Ladd et al. [47]
10	SpermX method	• Mock samples prepared by mixing saliva from female donor and semen from male donor with various dilutions ranging from 100:1 to 18,000:1 • Samples independently processed in three laboratories	• Freshly prepared mock samples	• SpermX recovered five times more male DNA than conventional DE • M: F ratio was 5.4 to 183 fold higher in SpermX method than conventional DE method	Sgueglia et al. [71]
11	Microfluidic device based on the hydrodynamic effect	• Mock samples prepared by mixing buccal epithelial cells and sperm cells • Epithelial and Sperm cells were diluted in PBS with a concentration of 1000 and 300 cells/µl	• Freshly prepared mock samples	• A throughput of 2 µl/ min • Percentage of male DNA in the mixed sample and the separated sample were 20.3 ± 12.1% and 94.0 ± 4.7% respectively	Liu et al. [53]

Table 4 (continued)

Sl. No.	Technique used	Type of sample tested	Condition of the sample	Recovery rate/ purity	Reference
12	DEPArray™ workflow	• *Proficiency test (PT) samples*: Single source semen swabs • *Mock samples*: Buccal epithelial cells + Sperm positive semen, Buccal epithelial cells + Sperm positive Semen + Whole Blood • *Post-coital Samples*: Vaginal swabs collected at 12, 24, 48, 72, and 96 h post-coitus.	• Proficiency test samples were ~ 15 years old • Mock samples and post-coital samples were processed freshly	• 175 to 238 spermatozoa recovered from PT samples having DNA concentrations between 0.58 and 0.79 ng/µL • 19 separate single sperm cell recoveries, 17 two sperm cell recoveries, and 14 three-sperm cell recoveries in mock samples • In post-coital samples, mixtures were found in 4/5 samples; DEPArray™ processed samples were easily interpreted due to the average peak height of the male contributors between 321 and 967 rfu	Williamson et al. [88]
13	Sperm Hy-Liter™ staining kit in conjugation with FACS	• Mock samples prepared by mixing buccal epithelial cells and sperm cells to obtain a sperm/epithelial ratio of 1:5, 1: 10, 1: 50, 1: 100, and 1: 500	• Freshly prepared mock samples	• 1, 3, 5 and 10 ratio of sperm and epithelial cells were separated • An average of 50%, 80%, 93.16% and 94.74% of the expected male alleles were observed for each respective subsample of 1, 3, 5 and 10 cells.	Fokias and Bekaert, [23]
14	Laser Capture Microdissection	• Mock samples prepared by combining 25 µl of the epithelial cell pellet working solution with 10 µl of the 1: 10 semen working solution	• Freshly prepared mock samples	• 300, 150, or 75 sperm and epithelial cells were dissected • All samples containing 300 LMD sperm displayed all 10 loci of the sperm donor • Samples containing 150 sperm exhibited on average 96 ± 3% of the male donors' alleles and samples containing 75 sperm cells displayed on average 72± 12% of the male donors' alleles	Sanders et al. [66]
15	Staining with Sperm Hy-Liter™, image processing by AxioVision Commander module followed by Laser pressure catapulting (LPC)	• Mock samples prepared by mixing Sperm cells and epithelial cells in the ratio 1:2, 1:10, and 1:50 • Pure semen smear and post-coital vaginal swab smears were also used	• Freshly prepared mock and post-coital samples	• 200, 100, 75, 50, 40, 30, 20, and 10 Sperm cells were collected by LPC • Full profile obtained with ≥ 20 isolated Spermatozoa	Vandewoestyne et al. [84]

Table 4 (continued)

Sl. No.	Technique used	Type of sample tested	Condition of the sample	Recovery rate/ purity	Reference
16	Magnetic bead conjugated with acrosin binding protein (ACRBP) antibody	• Mixed samples comprising three ratios prepared in a 100 µl sperm cell suspension (10^3 cells/ ml) and a 100 µl female buccal epithelial cell suspension (10^3, 10^4, or 10^5 cells/ ml) • Dried vaginal swabs from sexual assault cases	• Freshly prepared mock sexual assault samples • Vaginal swabs from sexual assault cases stored in a dry environment for > 6 months	• Average recovery rates 79%, 65%, and 31% in three mixed samples at 1:1, 1:10, and 1:100 male and female component ratios • Success rate of the five dried vaginal swabs was 60% for dried vaginal swabs stored for > 6 months	Li et al. [52]
17	MACSprep™ Forensic Sperm Micro-Bead Kit	• Mock sexual assault samples were prepared by adding 20µl of diluted sperm (1:200) on vaginal swabs	• Freshly prepared mock sexual assault samples used within 14 days	• Purity of Sperm fractions ranged from 54 to 96% • 10% higher purity compared to other techniques tested	Grosjean et al. [33]

Other techniques

A unique oligosaccharide SLeX, (NeuAcα2-3Galß1–4(Fucα1–3) GlcNAc) has been identified to be present in sperm which helps in binding with the egg cells. Another peripheral protein β1–4 galactosyltransferase 1 (B4GAL-T1) also plays a crucial role in the binding of sperm-oocyte. In this regard, Inci et al. [41] integrated SLeX and microfluidic technology for capturing sperm cells. After capturing, the sperm cells were treated with tris(2-carboxyethyl)phosphine (TCEP) in triton X-100 to lyse cells on-chip. This technique resulted in 70–92% sperm capture efficiency and 60–92% reduction in non-sperm fraction. Thus, it presents a next-generation differential extraction technology for rapid, reliable, accurate, user-friendly separation of sperm and non-sperm material from sexual assault evidence. Another study evaluated the MACSprep forensic sperm MicroBead kit which categorically retains spermatozoa by specific antibodies coupled to magnetic beads. MACSprep technique was found to generate ~ 10 times purer result in comparison to the Erase system with 1: 200 sperm dilution [33]. Besides, a combination of MACS and FACS has also been found useful in isolating a single sperm cell from forensic mixture samples which can be used in casework samples [92].

Advanced analytical techniques

Manipulation at the sampling and DNA isolation stage is crucial in successfully generating a male DNA profile from mixed samples as discussed above. However, forensic DNA analysis has made significant progress in the form of other analytical techniques such as the inception of low-volume PCR, the use of highly sensitive multiplexing kits, and the application of probabilistic genotyping software for deconvulating mixed DNA profiles.

Low volume PCR (LV-PCR)

Nowadays the single-cell genomics approach has gained importance. The routine PCR process with the recommended volume might not be effective in generating a successful DNA profile. In most of the sexual assault cases, the number of sperm cells is significantly low in comparison to the vaginal epithelial cells, and the M: F ratio varies widely in the varied nature of the cases. Though cell selection and cell picking techniques have been found useful, the generation of a complete DNA profile is dependent on a successful PCR process owing to the smaller number of sperm cells available for amplification. In this regard, on-chip low-volume PCR has been found effective in generating a complete STR profile using an Identifiler® kit when 30 sperm cells were taken for analysis. However, the percentage of obtaining a complete

profile was reduced to 80% and 90% when 15 and 20 sperm cells were selected respectively [49]. PCR mixture containing 4.2 µl of reaction mixture, 2.2 µl of primer mix, and 1 unit of AmpliTaq gold DNA polymerase has also been found to be effective in an efficient and affordable alternative in the analysis of mixed samples for forensic DNA purposes [50]. 0.75 µl of Identifiler (Applied Biosystems) PCR master mix was also found to be useful in DNA profiling of mixed samples when the cells are selected by a combination of fluorescence in situ hybridization (FISH) and laser microdissection [22].

Highly sensitive multiplex kits

After the inception of expanded CODIS on 1st January 2017 [35], several new generation multiplexing kits have been developed by different kit manufacturers for simultaneous amplification of more than 20 autosomal STR markers. Most of these multiplex kits are highly robust, sensitive towards low quantities of DNA, have better discrimination power with high M: F ratio, and high inhibitor tolerance capability. The new generation multiplex kits and their respective resolution powers of M: F ratio are listed in Table 2. Thus, differential analysis and cell selection techniques followed by the use of highly sensitive new-generation multiplex kits provide superior genotyping results in sexual assault mixed samples.

Probabilistic genotyping systems

Attribution of potential contributors in a mixed DNA profile is a major challenge for any forensic DNA laboratory. Probabilistic genotyping systems are of great use in predicting the probability of observing a DNA profile as a potential contributor in a single or mixed profile. The semi-continuous methods of probabilistic genotyping systems incorporate the probability of drop-out or drop-in while assigning the possible contributors. On the contrary, continuous methods consider the underlying behavior of peak heights while making assumptions [17]. The application of probabilistic genotyping systems has significantly improved the mixture interpretation following the guidelines set by SWGDAM, ISFG, and the UK forensic regulator [18]. Various systems have been developed and evaluated over the years for their aptness in deconvulating the mixed genotypes and have been found of suitable use in sexual assault sample investigations. Some of the useful probabilistic genotyping software include EuroForMix (http://www.euroformix.com/), DNAStatistX (developed by the Netherlands Forensic Institute), STRmix™ (https://www.strmix.com/) [26], MaSTR™ (SoftGenetics, PA 16803, USA) [38], TrueAllele® casework system (Cybergenetics, PA 15213, USA), Forensic statistical tool (FST) (in house forensic software package developed by the University of New Haven) [58] and many others.

Conclusion

Since the development of the differential extraction technique by Gill et al. [27], forensic DNA analysis of sexual assault cases has witnessed significant advancements over the years. The use of DTT, β-mercapto ethanol, and trizol supplemented with tris(2-carboxyethyl)phosphine (TCEP) has shown complete lysis of sperm cells in a mixture sample. Further, an increase in pH of the lysis buffer to 8.5 and incubation of cells at 42°C for 30 min has shown a significant increase in the recovery of sperm DNA. Though several chemicals and physical parameters have been standardized for the efficient lysis of sperm cells from a mixture, the carryover of sperm DNA into the non-sperm fraction (NSF) has been a major problem. In this regard, automated and semi-automated differential techniques have been developed such as Hamilton AutoLys STAR system, Automated Differex™ system, Sampletype I-sep DL, QIAcube HID differential washing station and BioRobot EZ1. On-chip sperm lysis methods have also gained importance in the last two decades. But the significant advancements witnessed in the form of sperm cell separation techniques either by using filtration techniques, immunological and affinity techniques, LCM technique, DEPArray™ systems, fluorescence-activated cell sorting, acoustic trapping, pressure-cycling, on-chip method, and holographic optical trapping (HOT). Some of the techniques have established themselves for ready use in sexual assault cases whereas some of them are having conceptual development. In this regard, the technology readiness level (TRL) of all the available techniques is summarized in Table 3.

Owing to the increasing trend in sexual assault cases, an efficient cell differentiation technique is the need of the hour. As rightly mentioned by Butler [12], the forensic techniques should be in line with olympic slogan i.e., faster, higher, stronger. In this regard, none of the available techniques can be considered a gold standard technique for the absolute separation of sperm and non-sperm DNA from mixed samples. The cell separation efficiency always depends on the nature and condition of the biological samples. A comparative account of the different available techniques and their performance is given in Table 4. Thus, choosing a differential extraction strategy is always sample-dependent, technique-dependent, and analyst's preference.

Acknowledgement The authors are highly acknowledged to Vice Chancellor, NFSU and Campus Director, NFSU, Delhi Campus to provide infrastructure to carry out this work.

Author contribution HRD is the sole author responsible for the study conception, material preparation, data collection, analysis, writing and reviewing the manuscript.

Funding None.

Data availability Not available.

Declarations

Ethical approval Not applicable.

Research involving human participants and/or animals Not applicable.

Informed consent Not applicable.

Conflict of interest The author declare no competing interests.

Reference

1. Alsalafi D, Goodwin W (2019) Capturing spermatozoa for STR analysis of sexual assault cases using antisperm antibodies. Forensic Sci Int: Genet Suppl Ser 7:707–710. https://doi.org/10.1016/j.fsigss.2019.10.146
2. Amaral A, Castillo J, Ramalho-Santos J, Oliva R (2014) The combined human sperm proteome: cellular pathways and implications for basic and clinical science. Hum Reprod Update 20:40–62
3. Andre B, Duterme C, Moer KV, Mertens-Strijthagen J, Jadot M, Flamion B (2011) Hyal2 is a glycosylphosphatidylinositol-anchored, lipid raft-associated hyaluronidase. Biochem Biophys Res Commun 411:175–179. https://doi.org/10.1016/j.bbrc.2011.06.125
4. Apostolov A (2014) Differentiation of mixed biological traces in sexual assaults using DNA fragment analysis. Biotechnol Biotechnol Equip 28:301–305. https://doi.org/10.1080/13102818.2014.909171
5. Auka N, Valle M, Cox BD, Wilkerson PD, Cruz TD, Reiner JE, Seashols-Williams SJ (2019) Optical tweezers as an effective tool for spermatozoa isolation from mixed forensic samples. PLoS ONE. https://doi.org/10.1371/journal.pone.0211810
6. Axler-DiPerte G, Orans S, Singh A, Caragine T, Prinz M, Budimlja ZM (2011) Comparison and optimization of DNA recovery from sperm vs. epithelial cells using laser capture microdissection technology and an immunofluorescent staining system. Forensic Sci Int Genet Suppl Ser 3:e224–e225. https://doi.org/10.1016/j.fsigss.2011.08.112
7. Bakdash A, Kumar S, Gautam KA, Mishra VC (2018) Use of flow cytometry in forensic medicine: Current scenario and future prospects. J Forensic Leg Med 60:42–44. https://doi.org/10.1016/j.jflm.2018.09.010
8. Bellastella G, Cooper G, Battaglia M, Strose A, Torres I, Hellenkemper B, Soler C, Sinii A (2010) Dimensions of human ejaculated spermatozoa in papanicolaou-stained seminal and swim-up smears obtained from the integrated semen analysis system (ISAS((R))). Asian J Androl 12:871–879
9. Berendsen JTW, Eijkel JCT, Wetzels AM, Segerink LI (2019) Separation of spermatozoa from erythrocytes using their tumbling mechanism in a pinch flow fractionation device. Microsystems Nanoengineering 5:24. https://doi.org/10.1038/s41378-019-0068-z
10. Bienvenue JM, Duncalf N, Marchiarullo D, Ferrance JP, Landers JP (2006) Microchip-based cell lysis and DNA extraction from sperm cells for application to forensic analysis. J Forensic Sci 51:266–273. https://doi.org/10.1111/j.1556-4029.2006.00054.x
11. Bogas V, Bento AM, Serra A, Brito P, Lopes V, Sampaio L, Gouveia N, Cunha P, Balsa F, São-Bento M, Porto MJ (2017) Validation of sampletype I-sep DL for differential extraction and purification with prepfiler express in the automate express DNA extraction system. Forensic Sci Int: Genet Suppl Ser 6:e353–e354. https://doi.org/10.1016/j.fsigss.2017.09.113
12. Butler JM (2015) The future of forensic DNA analysis. Philos Trans R Soc Lond B Biol Sci 370:20140252. https://doi.org/10.1098/rstb.2014.0252
13. Champroux A, Torres-Carreira J, Gharagozloo P, Drevet JR, Kocer A (2016) Mammalian sperm nuclear organization: resiliencies and vulnerabilities. Basic Clin Androl 26:17. https://doi.org/10.1186/s12610-016-0044-5
14. Chen J, Feng W, Zhan F (2019) Separation and forensic identification of sperm from cell mixtures using anti-hLCN6 monoclonal antibody coupled magnetic beads. Sheng Wu Gong Cheng Xue Bao 35:150–158. https://doi.org/10.13345/j.cjb.180075
15. Chen J, Kobilinsky L, Wolosin D, Shaler R, Baum H (1998) A physical method for separating spermatozoa from epithelial cells in sexual assault evidence. J Forensic Sci 43:114–118
16. Clark CP, Xu K, Scott O, Hickey J, Tsuei AC, Jackson K, Landers JP (2019) Acoustic trapping of sperm cells from mock sexual assault samples. Forensic Sci Int Genet 41:42–49. https://doi.org/10.1016/j.fsigen.2019.03.012
17. Coble MD, Bright JA (2019) Probabilistic genotyping software: An overview. Forensic Sci Int Genet 38:219–224. https://doi.org/10.1016/j.fsigen.2018.11.009
18. Coble MD, Buckleton J, Butler JM, Egeland T, Fimmers R, Gill P, Gusmão L, Guttman B, Krawczak M, Morling N, Parson W, Pinto N, Schneider PM, Sherry ST, Willuweit S, Prinz M (2016) DNA Commission of the International Society for Forensic Genetics: Recommendations on the validation of software programs performing biostatistical calculations for forensic genetics applications. Forensic Sci Int Genet 25:191–197. https://doi.org/10.1016/j.fsigen.2016.09.002
19. Court DS (2021) The Y chromosome and its use in forensic DNA analysis. Emerg Top Life Sci 5:427–441. https://doi.org/10.1042/ETLS20200339
20. Elliott K, Hill DS, Lambert C, Burroughes TR, Gill P (2003) Use of laser microdissection greatly improves the recovery of DNA from sperm on microscope slides. Forensic Sci Int 137:28–36
21. Ensenberger MG, Lenz KA, Matthies LK, Hadinoto GM, Schienman JE, Przech AJ, Morganti MW, Renstrom DT, Baker VM, Gawrys KM, Hoogendoorn M, Steffen CR, Martín P, Alonso A, Olson HR, Sprecher CJ, Storts DR (2016) Developmental validation of the PowerPlex® Fusion 6C system. Forensic Sci Int: Genet 21:134–144. https://doi.org/10.1016/j.fsigen.2015.12.011
22. Feng L, Li C, Han J, Xu C, Hu L (2014) Isolating cells from female/male blood mixtures using florescence in situ hybridization combined with low volume PCR and its application in forensic science. Int J Legal Med. https://doi.org/10.1007/s00414-014-1103-0
23. Fokias K, Bekaert B (2022) Separation of sperm and epithelial cells based on fluorescence-activated cell sorting. Forensic Sci Int Genet Suppl Ser 8:239–241. https://doi.org/10.1016/j.fsigss.2022.10.048
24. Fonneløp AE, Johannessen H, Heen G, Molland K, Gill P (2019) A retrospective study on the transfer, persistence and recovery of sperm and epithelial cells in samples collected in sexual assault casework. Forensic Sci Int Genet 43:102153. https://doi.org/10.1016/j.fsigen.2019.102153
25. Garvin AM, Fischer A, Schnee-Griese J, Jelinski A, Bottinelli M, Soldati G, Tubio M, Castella V, Monney N, Malik N, Madrid M (2012) Isolating DNA from sexual assault cases: a comparison of standard methods with a nuclease-based approach. Invest Genet 3:25. https://doi.org/10.1186/2041-2223-3-25
26. Gill P, Benschop C, Buckleton J, Bleka O, Taylor D (2021) A review of probabilistic genotyping systems: EuroForMix, DNAStatistX and STRmix™. Genes (Basel) 12:1559. https://doi.org/10.3390/genes12101559
27. Gill P, Jeffreys AJ, Werrett DJ (1985) Forensic application of DNA fingerprints. Nature 318:577–579. https://doi.org/10.1038/318577a0

28. Goldstein MC, Cox JO, Seman LB, Cruz TD (2019) Improved resolution of mixed STR profiles using a fully automated differential cell lysis/DNA extraction method. Forensic Sci Res. https://doi.org/10.1080/20961790.2019.1646479
29. Gómez-Torres MJ, Sáez-Espinosa P, Manzano-Santiago P, Robles-Gómez L, Huerta-Retamal N, Aizpurua J (2022) Sperm adhesion molecule 1 (SPAM1) distribution in selected human sperm by hyaluronic acid test. Biomedicines 10:2553. https://doi.org/10.3390/biomedicines10102553
30. Green R, Elliott JL, Norona W, Go F, Nguyen VT, Ge J, Short ML, Mulero JJ, Zhong C (2021) Developmental validation of VeriFiler™ Plus PCR Amplification Kit: A 6-dye multiplex assay designed for casework samples. Forensic Sci Int Genet 53:102494. https://doi.org/10.1016/j.fsigen.2021.102494
31. Greenspoon SA, Scarpetta MA, Drayton ML, Turek SA (1998) QIAamp spin columns as a method of DNA isolation for forensic casework. J Forensic Sci 43:1024–1030
32. Grgicak CM, Sipes D, Leisy C, Word CJ, Cotton RW, 55th Annual American Academy of Forensic Sciences Meeting (2003) Comparative analysis of the DNA IQTM and QiAamp® DNA extraction kits for the processing of forensic evidentiary samples. Proceedings of the 55th Annual American Academy of Forensic Sciences Meeting; 2003 Feb 17–22; Chicago, IL. Colorado Springs, CO: American Academy of Forensic Sciences, 26–27
33. Grosjean F, Favre M, Castella V (2023) Comparison between MACSprep™ forensic sperm microbead kit and Erase Sperm Isolation kit for the enrichment of sperm fractions recovered from sexual assault samples. Int J Legal Med 137:267–278. https://doi.org/10.1007/s00414-022-02861-7
34. Hamatani T, Tanabe K, Kamei K, Sakai N, Yamamoto Y, Yoshimura Y (2000) A monoclonal antibody to human SP-10 inhibits in vitro the binding of human sperm to hamster oolemma but not to human Zona pellucida. Biol Reprod 62:1201–1208. https://doi.org/10.1095/biolreprod62.5.1201
35. Hares DR (2015) Selection and implementation of expanded CODIS core loci in the United States. Forensic Sci Int Genet 17:33–34. https://doi.org/10.1016/j.fsigen.2015.03.006
36. Hennekens CM, Cooper ES, Cotton RW, Grgicak CM (2013) The effects of differential extraction conditions on the premature lysis of spermatozoa. J Forensic Sci 58:744–752. https://doi.org/10.1111/1556-4029.12098
37. Heydari A, Targhi MZ, Halvaei I, Nosrati R (2023) A novel microfluidic device with parallel channels for sperm separation using spermatozoa intrinsic behaviors. Sci Rep 13:1185. https://doi.org/10.1038/s41598-023-28315-7
38. Holland MM, Tiedge TM, Bender AJ, Gaston-Sanchez SA, McElhoe JA (2022) MaSTR™: an effective probabilistic genotyping tool for interpretation of STR mixtures associated with differentially degraded DNA. Int J Legal Med 136:433–446. https://doi.org/10.1007/s00414-021-02771-0
39. Huang J, Chen H, Li N, Zhao Y (2023) Emerging microfluidic technologies for sperm sorting. Eng Regeneration 5:161–169. https://doi.org/10.1016/j.engreg.2023.02.001
40. Hudlow WR, Buoncristiani MR (2012) Development of a rapid, 96-well alkaline based differential DNA extraction method for sexual assault evidence. Forensic Sci Int Genet 6:1–16. https://doi.org/10.1016/j.fsigen.2010.12.015
41. Inci F, Ozen MO, Saylan Y, Miansari M, Cimen D, Dhara R, Chinnasamy T, Yuksekkaya M, Filippini C, Kumar DK, Calamak S, Yesil Y, Durmus NG, Duncan G, Klevan L, Demirci U (2019) A novel on-chip method for differential extraction of sperm in forensic cases. Adv Sci 5:1800121. https://doi.org/10.1002/advs.201800121
42. Jirovec A, Flaman A, Godbout E, Serrano D, Werier J, Purgina B, Diallo JS (2024) Immune profiling of dedifferentiated liposarcoma and identification of novel antigens for targeted immunotherapy. Sci Rep 14:11254. https://doi.org/10.1038/s41598-024-61860-3
43. Kato Y, Kumar S, Lessard C, Bailey JL (2021) ACRBP (Sp32) is involved in priming sperm for the acrosome reaction and the binding of sperm to the zona pellucida in a porcine model. PLoS ONE 16:e0251973. https://doi.org/10.1371/journal.pone.0251973
44. Khouzestani A, Hojjat Y, Tavalaee M, Sadeghian H, Nasr-Esfahani MH (2023) Enhancing the accuracy of measuring DEP force applied on cells by considering the friction effect. Biosensors 13:540. https://doi.org/10.3390/bios13050540
45. Klein SB, Buoncristiani MR (2017) Evaluating the efficacy of DNA differential extraction methods for sexual assault evidence. Forensic Sci Int Genet 29:109–117. https://doi.org/10.1016/j.fsigen.2017.03.021
46. Kraemer M, Prochnow A, Bussmann M, Scherer M, Peist R, Steffen C (2017) Developmental validation of QIAGEN Investigator124plex QS Kit and Investigator124plex GO! Kit: Two 6-dye multiplex assays for the extended CODIS core loci. Forensic Sci Int Genet 29:9–20. https://doi.org/10.1016/j.fsigen.2017.03.012
47. Ladd C, Carita E, Pagliaro E, Garvin A, Crumbie A, Lee H (2006) Development of a high-throughput method to isolate sperm DNA in sexual assault cases (NIJ award 2003-IJ-CX-K013, document 215339), chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj, https://www.ojp.gov/pdffiles1/nij/grants/215339.pdf. Accessed 9 Feb 2024
48. Laurell T, Petersson F, Nilsson A (2006) Chip integrated strategies for acoustic separation and manipulation of cells and particles. Chem Soc Rev 36:492–506
49. Li C, Han J, Ren W, Ji A, Xu X, Hu L (2011a) DNA Profiling of spermatozoa by laser capture microdissection and low volume-PCR. PLoS ONE 6:e22316. https://doi.org/10.1371/journal.pone.0022316
50. Li C, Wang G, Li W, Huang J, Ji A, Hu L (2011b) New cell separation technique for the isolation and analysis of cells from biological mixtures in forensic caseworks. Croatian Med J 52:293–308. https://doi.org/10.3325/cmj.2011.52.293
51. Li XB, Wang QS, Feng Y, Ning SH, Miao YY, Wang YQ, Li HW (2014) Magnetic bead-based separation of sperm from buccal epithelial cells using a monoclonal antibody against MOSPD3. Int J Legal Med 128:905–911. https://doi.org/10.1007/s00414-014-0983-3
52. Li X, Xu F, Zheng J, Sun M, Zhu X, Lv P, Du Z, Zhang X, Yao J (2022) Magnetic bead-based separation of sperm cells from semen-vaginal fluid mixed stains using an anti-ACRBP antibody. Int J Legal Med 137:511–518. https://doi.org/10.1007/s00414-022-02917-8
53. Liu W, Chen W, Liu R, Ou Y, Liu H, Xie L, Lu Y, Li C, Li B, Cheng J (2015) Separation of sperm and epithelial cells based on the hydrodynamic effect for forensic analysis. Biomicrofluidics 9:044127. https://doi.org/10.1063/1.4928453
54. Lounsbury JA, Nambiar SM, Karlsson A, Cunniffe H, Norris JV, Ferrance JP, Landers JP (2014) Enhanced recovery of spermatozoa and comprehensive lysis of epithelial cells from sexual assault samples having a low cell counts or aged up to one year. Forensic Sci Int Genet 8:84–89. https://doi.org/10.1016/j.fsigen.2013.06.015
55. Ludeman MJ, Zhong C, Mulero JJ, Lagacé RE, Hennessy LK, Short ML, Wang DY (2018) Developmental validation of GlobalFiler™ PCR amplification kit: a 6-dye multiplex assay designed for amplification of casework samples. Int J Legal Med 132:1555–1573. https://doi.org/10.1007/s00414-018-1817-5
56. Marcińska M, Parys-Proszek A, Wróbel M (2022) Evaluation of the Investigator 26plex QS and SureID PanGlobal systems in forensic analysis and comparison with commonly used NGM PCR Amplification Kit. Probl Forensic Sci 129:93–103. https://doi.org/10.4467/12307483PFS.22.005.16306
57. Micó V, Zalevsky Z, Ferreira C, García J (2008) Superresolution digital holographic microscopy for three-dimensional samples. Opt Express 16:19260–19270. https://doi.org/10.1364/OE.16.019260

58. Mitchell AA, Tamariz J, O'Connell K, Ducasse N, Budimlija Z, Prinz M, Caragine T (2012) Validation of a DNA mixture statistics tool incorporating allelic drop-out and drop-in. Forensic Sci Int Genet 6:749–761. https://doi.org/10.1016/j.fsigen.2012.08.007
59. Ng HH, Lim ML, Hoe SY, Yong ZD, Ping YS, Ang HC, Syn CKC (2017) Modified differential DNA extraction to reduce processing time of sexual assault exhibits. Forensic Sci Int Genet Suppl Ser 6:e252–e254. https://doi.org/10.1016/j.fsigss.2017.09.094
60. Norris JV, Evander M, Horsman-Hall KM, Nilsson J, Laurell T, Landers JP (2009) Acoustic differential extraction for forensic analysis of sexual assault evidence. Anal Chem 81:6089–6095. https://doi.org/10.1021/ac900439b
61. Patil PS, Humbarwadi RS, Patil AD, Gune AR (2013) Immature germ cells in semen – correlation with total sperm count and sperm motility. J Cytol 30:185–189. https://doi.org/10.4103/0970-9371.117682
62. Ralf A, Montiel González D, Zandstra D, van Wersch B, Kousouri N, de Knijff P, Adnan A, Claerhout S, Ghanbari M, Larmuseau MHD, Kayser M (2023) Large-scale pedigree analysis highlights rapidly mutating Y-chromosomal short tandem repeats for differentiating patrilineal relatives and predicting their degrees of consanguinity. Hum Genet 142:145–160. https://doi.org/10.1007/s00439-022-02493-2
63. Ren Z, Huang X, Lv Q, Lei Y, Shi H, Wang F, Wang M (2022) High expression of B4GALT1 is associated with poor prognosis in acute myeloid leukemia. Front Genet 13:882004. https://doi.org/10.3389/fgene.2022.882004
64. Roszkowski M, Mansuy IM (2021) High efficiency RNA extraction from sperm cells using guanidinium thiocyanate supplemented with tris(2-carboxyethyl)phosphine. Front Cell Dev Biol 9:648274. https://doi.org/10.3389/fcell.2021.648274
65. Samuel R, Feng H, Jafek A, Despain D, Jenkins T, Gale B (2018) Microfluidic-based sperm sorting & analysis for treatment of male infertility. Transl Androl Urol 7:S336–S347. https://doi.org/10.21037/tau.2018.05.08
66. Sanders CT, Sanchez N, Ballantyne J, Peterson DA (2006) Laser microdissection separation of pure spermatozoa from epithelial cells for short tandem repeat analysis. J Forensic Sci 51:748–757
67. Sarbandi IR, Lesani A, Zand MM, Nosrati R (2021) Rheotaxis-based sperm separation using a biomimicry microfluidic device. Sci Rep 11:18327. https://doi.org/10.1038/s41598-021-97602-y
68. Schoell WMJ, Klintschar M, Mirhashemi R, Pertl B (1999) Separation of sperm and vaginal cells with flow cytometry for DNA typing after sexual assault. Obstet Gynecol 94:623–627. https://doi.org/10.1016/S0029-7844(99)00373-7
69. Schoell WMJ, Klintschar M, Mirhashemi R, Strunk D, Giuliani A, Bogensberger G, Pertl B (1999) Separation of sperm and vaginal cells based on ploidy, MHC class I -, CD45 -, and cytokeratin expression for enhancement of DNA typing after sexual assault. Cytometry 36:319–323
70. Schwerdtner G, Germann U, Cossu C (2017) The separation of male and female: A comparison of seven protocols (P). Forensic Sci Int: Genet Suppl Ser 6:e9–e11. https://doi.org/10.1016/j.fsigss.2017.09.021
71. Sgueglia JB, Holt H, Hanson E, Nichols J, Kalafut T, Khan M, Walsh T, Foley M, Brown H, Ballantyne J, Sinha SK (2023) Interlaboratory comparison of SpermX™ and conventional differential extractions indicated high male DNA recovery by the SpermX method. Forensic Sci 3:592–610. https://doi.org/10.3390/forensicsci3040043
72. Simone NL, Bonner RF, Gillespie JW, Emmert-Buck MR, Liotta LA (1998) Laser-capture microdissection: opening the microscopic frontier to molecular analysis. Trends Genet 14:272–276. https://doi.org/10.1016/S0168-9525(98)01489-9
73. Sinha SK, Brown H, Holt H, Khan M, Brown R, Sgueglia JB, Loftus A, Murphy G, Montgomery A (2022) Development and validation of a novel method SpermX™ for high throughput differential extraction processing of sexual assault kits (SAKs) for DNA analysis. Forensic Sci Int Genet 59:102690. https://doi.org/10.1016/j.fsigen.2022.102690
74. Sun J, Li M, Liu C, Zhang Y, Liu D, Liu W, Hu G, Jiang X (2012) Double spiral microchannel for label-free tumor cell separation and enrichment. Lab Chip 12:3952–3960. https://doi.org/10.1039/c2lc40679a
75. Sun M, Zheng J, Wang B, Yao J (2021) Sperm cell capture based on ABH antigen differences to separate two men in mixed seminal stains. Biomed Res Int 2021(7269237). https://doi.org/10.1155/2021/7269237
76. Timken MD, Klein SB, Kubala S, Scharnhorst G, Buoncristiani MR, Miller KWP (2019) Automation of the standard DNA differential extraction on the Hamilton AutoLys STAR system: a proof-of-concept study. Forensic Sci Int Genet 40:96–104. https://doi.org/10.1016/j.fsigen.2019.02.011
77. Tosi D, Sypabekova M, Bekmurzayeva A, Molardi C, Dukenbayev K (2022) Grating-based sensors. In: Optical fiber biosensors, device platforms, biorecognition, applications. 79–105. https://doi.org/10.1016/B978-0-12-819467-6.00004-4
78. Tsuji Y, Clausen H, Nudelman E, Kaizu T, Hakomori S, Isojima S (1988) Human sperm carbohydrate antigens defined by an antisperm human monoclonal antibody derived from an infertile woman bearing antisperm antibodies in her serum. J Exp Med 168:343–356. https://doi.org/10.1084/jem.168.1.343
79. Tsukada K, Asamura H, Ota M, Kobayashi K, Fukushima H (2006) Sperm DNA extraction from mixed stains using the Differex System. Int Congr Ser 1288:700–703. https://doi.org/10.1016/j.ics.2005.12.059
80. Tu Z, Peng J, Long X, Li J, Wu L, Huang K, Zhu X (2022) Sperm autoantigenic protein 17 predicts the prognosis and the immunotherapy response of cancers: a pan-cancer analysis. Front Immunother. https://doi.org/10.3389/fimmu.2022.844736
81. Valgren C, Edenberger E (2008) Evaluation of the Differex™ System. Forensic Sci Int: Genet Suppl Ser 1:78–79. https://doi.org/10.1016/j.fsigss.2007.08.008
82. Valle M, O'Brien B, Green TD, Reiner JE, Seashols-Williams S (2024) Droplet-based optical trapping for cell separation in mock forensic samples. J Forensic Sci 69:273–281. https://doi.org/10.1111/1556-4029.15379
83. Vandewoestyne M, Deforce D (2010) Laser capture microdissection in forensic research: a review. Int J Legal Med 124:513–521. https://doi.org/10.1007/s00414-010-0499-4
84. Vandewoestyne M, Van Hoofstat D, Van Nieuwerburgh F, Deforce D (2009) Automatic detection of spermatozoa for laser capture microdissection. Int J Legal Med 123:169–175. https://doi.org/10.1007/s00414-008-0271-1
85. Vasilescu SA, Khorsandi S, Ding l, Bazaz SR, Nosrati R, Gook D, Warkiani ME (2021) A microfluidic approach to rapid sperm recovery from heterogeneous cell suspensions. Sci Rep 11:7917. https://doi.org/10.1038/s41598-021-87046-9
86. Vuichard S, Borer U, Bottinelli M, Cossu C, Malik N, Meier V, Gehrig C, Sulzer A, Morerod ML, Castella V (2011) Differential DNA extraction of challenging simulated sexual-assault samples: a Swiss collaborative study. Invest Genet 2:11. https://doi.org/10.1186/2041-2223-2-11
87. White TJ, Rye MS, Tay JW (2022) Developmental validation of an efficient differential separation method incorporating the i-sep® DL spin column with high sperm DNA recovery for the processing of sexual assault samples. J Forensic Sci 67:1679–1689. https://doi.org/10.1111/1556-4029.15043
88. Williamson VR, Laris TM, Romano R, Marciano MA (2018) Enhanced DNA mixture deconvolution of sexual offense

samples using the DEPArray™ system. Forensic Sci Int Genet 34:265–276. https://doi.org/10.1016/j.fsigen.2018.03.001
89. Wu H, de Gannes MK, Luchetti G, Pilsner JR (2018) Rapid method for the isolation of mammalian sperm DNA. Biotechniques 58:6. https://doi.org/10.2144/000114280
90. Xu K, Clark CP, Poe BL, Lounsbury JA, Nilsson J, Laurell T, Landers JP (2019) Isolation of a low number of sperm cells from female DNA in a Glass–PDMS–Glass microchip via bead-assisted acoustic differential extraction. Anal Chem 91:2186–2191. https://doi.org/10.1021/acs.analchem.8b04752
91. Xu K, Yang L, Zhang L, Qi H (2020) Lack of AKAP3 disrupts integrity of the subcellular structure and proteome of mouse sperm and causes male sterility. Development 147:dev181057. https://doi.org/10.1242/dev.181057
92. Xu Y, Xie J, Chen R, Cao Y, Ping Y, Xu Q, Hu W, Wu D, Gu L, Zhou H, Chen X, Zhao Z, Zhong J, Li R (2016) Fluorescence- and magnetic-activated cell sorting strategies to separate spermatozoa involving plural contributors from biological mixtures for human identification. Sci Rep 6:36515. https://doi.org/10.1038/srep36515
93. Yamada M, Nakashima M, Seki M (2004) Pinched flow fractionation: continuous size separation of particles utilizing a laminar flow profile in a pinched microchannel. Anal Chem 76:5465–5471. https://doi.org/10.1021/ac049863r
94. Yoshida K, Sekiguchi K, Mizuno N, Kasai K, Sakai I, Sato H, Seta S (1995) The modified method of two-step differential extraction of sperm and vaginal epithelial cell DNA from vaginal fluid mixed with semen. Forensic Sci Int 72:25–33. https://doi.org/10.1016/0379-0738(94)01668-u
95. Zeginiadou T, Papadimas J, Mantalenakis S (2001) Acrosome reaction: methods for detection and clinical significance. Andrologia 32:335–343. https://doi.org/10.1046/j.1439-0272.2000.00359.x
96. Zhao XC, Wang L, Sun J, Jiang BW, Zhang EL, Ye J (2016) Isolating sperm from cell mixtures using magnetic beads coupled with an Anti-PH-20 antibody for forensic DNA analysis. PLoS ONE. https://doi.org/10.1371/journal.pone.0159401