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Evolution of Laboratory Techniques in Forensic Biology: From Conventional Methods to Modern Molecular Approaches

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Abstract

During a crime scene investigation biological techniques play very important role in forensic investigations by linking biological evidences to crime events. Traditional identification methods such as chemical & immunological tests have been widely used lab. But these techniques often suffer from many limitations such as low specificity, damaging the evidence or some sensitivity. Recent advances in molecular biology have introduced novel approaches such as RNA profiling & DNA methylation method for more accurate identification of collected samples. Here we summarize conventional & modern techniques used in forensic body fluid identification & discuss their applications in forensic investigations by establishing a connection between sample donors & actual criminal acts. Kind & source of bodily fluids discovered at a crime scene can provide crucial information for crime scene reconstruction. Numerous techniques for identifying bodily fluids have been developed for over a century including protein catalytic activity tests, spectroscopic techniques, chemical tests, immunological tests & microscopy. These traditional techniques for identifying samples are primarily reasonable & only apply to a single bodily fluid at a time. So it has recently been suggested that traditional body fluid identification techniques be replaced with a molecular genetics based approach that uses RNA profiling or DNA methylation detection. The specificity & sensitivity of a number of RNA markers & tDMRs (Tissue-specific Differentially Methylated Regions) that are unique to bodily fluids that are relevant to forensics have been determined using a variety of samples. In this review, we give a summary of the state of the art & the latest advancements in forensic body fluid identification & talk about how it might be used in forensic cases.

Introduction

Biological evidences collected from crime scenes provide critical information for forensic investigations. The identification of biological materials such as blood, semen, saliva, & other bodily samples allows investigators to reconstruct events & establish associations between individuals & criminal activities (An, J. H et al., 2012). The development of DNA Profiling is latest technique that has revolutionized forensic science by enabling the identification of individuals with a high grade of accuracy. Despite the success of DNA profiling, determining the type of biological fluid present at a crime scene remains equally important (An, J. H et al., 2012). Different body secretions provide specific information about the nature of the crime. For example, the presence of blood may indicate physical violence whereas semen or vaginal secretions may suggest sexual assault (An, J. H et al., 2012). Reliable identification of body secretions is an essential component of forensic biology. Forensic laboratories have relied on chemical, enzymatic & immunological tests to detect body fluids. These methods often suffer from limitations such as cross reactivity, low sensitivity & the destruction of biological material. Recent advances in molecular biology have introduced new approaches based on RNA & epigenetic markers that allow more accurate & specific identification of biological samples. All kinds of basic science such as Biology, Chemistry & physics involved to investigate the crime scene & evidence identification. Such as biology helps in DNA profiling, entomological forensics & teeth or blood patterns. Chemistry helps in basic tests such as immunological, Kastle-Mayer & luminol test. Physics helps in detection of liquids with specific wave length UVs.

In forensic investigations DNA typing of biological evidence is important because it establishes whether a suspect may be connected to a specific crime. This is predicated on the idea that, aside from identical twins, every person has a distinct DNA profile (An et al., 2012). DNA testing is crucial for both identifying possible suspects and clearing wrongfully convicted people who enhance the justice system's dependability and equity (Ballantyne, 2013). Additionally the identification and description of biological materials found at crime scenes offer important information for reconstructing criminal incidents. Establishing a link between people and the assumed offense is made easier by identifying the kind and source of such evidence. For example the finding of semen or vaginal fluid may indicate sexual activity or assault. Same as the presence of bloodstains may indicate events like physical assault violent struggle or homicide. As a result the examination of bodily fluids greatly aids in comprehending the order and character of events at a crime scene (Bauer, 2007).

Biological stains such as blood, semen, saliva, vaginal secretions, urine, and sweat are some of the most common types of evidence found at crime scenes. In many cases, these fluids are hard to see and need special light sources or chemical reagents to be found. Possible testing is usually the first step in the analytical process. It gives a first idea of what the substance is that. After that confirmatory testing is done to make sure the biological material identity and origin are correct or not (Ballantyne, 2013). In the last hundred years a lot of different ways to analyze body fluids for forensic purposes have been made. Chemical assays, immunological techniques, protein based catalytic activity tests, spectroscopic analyses and microscopic methods are all used to find and describe biological evidence. Commonly used as preliminary screening tools because they can quickly and accurately find things possible tests like luminol and the Kastle_Meyer test for blood (Bauer & Patzelt, 2008). Confirmatory methods, on the other hand give clear proof of certain body fluids. For example the microscopic identification of spermatozoa is widely accepted as proof of semen detection (Ballantyne, 2013). Even though there are used many catalytic & enzymatic or immunological tests used in both presumptive and confirmatory testing have some problems. These may involve reduced specificity or constrained sensitivity, degradation of biological specimen instability of target biomolecules and potential incompatibility with subsequent DNA profiling methodologies. These kinds of problems show how important it is for forensic methods to keep getting better so that biological evidence analysis is always accurate and reliable (Bauer & Patzelt, 2008).

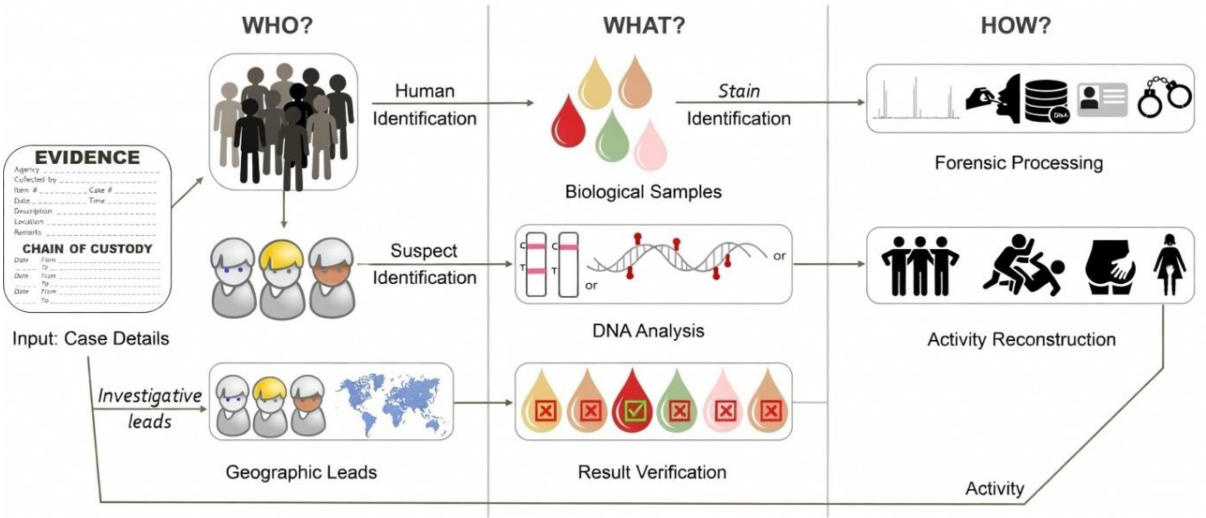

Figure.1: Systematic method of forensic investigation

Recent progress in forensic genetics has resulted in the formulation of numerous fresh methodologies for biological fluid identification particularly focusing on the examination of messenger RNA (mRNA) microRNA (miRNA) expression profiles and DNA methylation patterns (Butler, 2015). Methods that use tissue-specific mRNA and miRNA expression have been very successful mostly because they are very specific to different biological fluids that are important for forensic workout (Caballero & Viberg, 2019). However the practical implementation of RNA-based techniques is frequently impeded by the intrinsic instability of RNA molecules such as ribonucleases are omnipresent and can swiftly degrade mRNA. Also RNA typing usually needs a bigger sample of biological material unless RNA is extracted with DNA at the same time. DNA methylation-based assays on the other hand, have become a promising option. These techniques depend on identifying differential methylation patterns among diverse cell and tissue types providing elevated specificity and integration with current forensic workflows (Coble & Butler, 2010; Ballantyne, 2013).
Now well will review examination of both traditional and modern techniques employed for body fluid identification in forensic laboratories rigorously assessing their respective advantages and drawbacks. It also talks about new methods that have been made in the last ten years such as mRNA-based or miRNA-based and DNA methylation-based methods. These are big steps forward in making forensic analyses more accurate and reliable.

Biological Techniques
Catalytic assays that use the enzymatic activity of alpha-amylase are often used to find saliva. Saliva has a lot of this enzyme which breaks down polysaccharides into simpler sugar molecules (Gunn; Neumann; & Reber., 2019). The starch_iodine test is one of the oldest ways to find alpha-amylase. In this method, iodine reacts with starch to make a purple_black color that is easy to see. But when amylase is present, the starch breaks down which makes the color less intense (Hanson & Ballantyne., 2010). Another common method is the Phadebas® test, which uses a synthetic substrate called amylopectin procion red instead of the usual starch iodine system (Heaton et al., 2019). This assay is beneficial because it is cheap and very sensitive. Still it isn't completely specific because false positive results have been seen when hand creams, facial lotions, urine, and fecal matter are present. These substances may also show amylase like activity (Hsieh et al., 2018).
There are two types of alpha-amylase in the human body AMY which is mostly found in saliva breast milk and sweat and AMY2 which is found in the pancreas, semen and vaginal secretions. AMY1 is the most common form in saliva but both isoenzymes work in the same way. The identification of alpha-amylase activity offers merely presumptive evidence for the existence of saliva as it is not exclusively indicative of this bodily fluid (Hua et al., 2022). Immunological

tests that use specific antibody-antigen interactions are also very common in forensic investigations along with enzymatic assays. The OneStep ABAcard® HemaTrace test is a good example. It is an immuno-chromatographic assay that can find human blood (Irwin, 2016). This test works by having human hemoglobin stick to a moving monoclonal anti-human hemoglobin antibody. When an antibody-antigen complex forms it moves along an absorbent membrane to the test zone. There, the appearance of a pink dye means the test is positive. The test is very sensitive and can find human hemoglobin levels as low as 0.05 g/ml (Klein et al., 2021). The evidence shows that the OneStep ABAcard® HemaTrace test is a very sensitive and easy to use test for finding human blood. It can be used in labs and at crime scenes. To confirm the presence of semen, assays that look for prostate-specific antigen (PSA) are often used along with looking for spermatozoa under a microscope (Ladd et al., 2019). The male prostate gland makes a lot of PSA, and it can even be found in the semen of men who do not make sperm (An et al., 2012). The OneStep ABAcard® PSA is one of the most popular commercial antibody-antigen-based test kits for finding PSA. This assay employs a mobile monoclonal anti-human PSA antibody that selectively binds to PSA and traverses a test strip, generating a visible line where the antigen–antibody complex aggregates (Lee et al., 2020). Studies have shown that PSA can also be found in small amounts in other body fluids, like breast milk and female urine which could make it less specific (Li, Wang, & Ma, 2014). MicroRNA (miRNA) molecules have recently become promising biomarkers for identifying body fluids. miRNAs are brief non-coding RNA sequences that modulate gene expression and display tissue-specific expression profiles. Their small size and natural stability make them less likely to break down than mRNA which makes them great for studying old or damaged forensic samples. Recent studies have pinpointed various miRNA markers that can consistently differentiate among distinct bodily fluids, thereby improving the accuracy of forensic analyses (Lindenbergh & Sijen, 2016). Ribonucleases make RNA unstable by nature. Recent studies have shown that RNA taken from certain forensic stains can be surprisingly stable in some situations (Ludwig et al., 2017). Whole genome expression analyses of aged blood and saliva stains have identified messenger RNA (mRNA) markers with stable expression profiles enabling reliable detection even after 180 days of storage. Some of these markers have been successfully amplified from blood stains that were over 16 years old indicating the long-term utility of mRNA-based tests (Maher, 2019). One of the best things about using mRNA profiling to find body fluids is that you can get DNA and mRNA from the same sample. This is very helpful in forensic work where there isn't always a lot of biological material to work with. You can tell what kind of body fluid it is and do genetic profiling with just one stain (Virkler & Lednev, 2009).

To make it easier to separate mRNA and DNA from the same biological stain at the same time several advanced methods have been created. This improves efficiency and protects limited forensic material (McCord & Schwarzhoff, 2001). These methods can tell you what kind of body fluid is in the sample and also confirm the donor's genetic identity (Mori et al., 2023). Another big benefit of mRNA profiling is that it can find many body fluids in one multiplex reaction giving you a complete picture of how many genes are expressed at the same time. Different multiplex assays that use reverse transcription endpoint polymerase chain reaction (RT-PCR) and real-time quantitative reverse transcription PCR (qRT-PCR) have been reported. These assays use markers that are unique to different body fluids (Nechvatal & Budowle, 2019). Since a lot of mRNA transcripts are not completely tissue-specific and show different levels of expression qRT- PCR is especially helpful for measuring relative gene expression across samples. On the other hand, endpoint PCR is a good way to find transcripts that are only expressed in certain tissues. This makes it a reliable way to confirm the presence of certain body fluids (Nechvatal & Budowle, 2019).

Recent epigenomic studies have revealed that DNA displays tissue specific methylation patterns leading to inquiries into the application of differential DNA methylation for body fluid identification in forensic science (Nishita et al., 2021). DNA methylation which takes place at the 5′-position of cytosine residues in CpG dinucleotide is a well-known way to control gene

expression by changing the structure of chromatin (Oldoni & van Oorschot, 2018). Different types of cells have different methylation patterns and certain parts of chromosomes called tissue-specific differentially methylated regions (tDMRs), have different methylation patterns depending on the cell or tissue they come from (Pérez et al., 2020). Evaluating the DNA methylation status at specific CpG sites within tDMRs can offer a dependable method for identifying the tissue or cell type of origin for a DNA sample. For instance. Frumkin et al. (2018) discovered 15 genomic loci demonstrating differential methylation in diverse biological materials such as venous and menstrual blood, saliva, semen, skin epidermis, urine and vaginal secretions. The researchers used methylation sensitive restriction enzyme PCR (MSRE-PCR) to look at markers in their study. This technique entails the digestion of sample DNA using methylation sensitive restriction enzymes succeeded by multiplex PCR aimed at specific genomic loci utilizing fluorescence labeled primers. Capillary electrophoresis is then used to separate the amplified products and signals are automatically detected. This makes it possible to accurately determine tissue specific methylation patterns (Pereira et al., 2018).
The assay can be easily added to st&ard procedures used in forensic labs like DNA typing with short T&em repeat (STR) & it was able to find source tissues in 51 DNA samples from blood or saliva may be semen & skin (An, J. H et al., 2012).

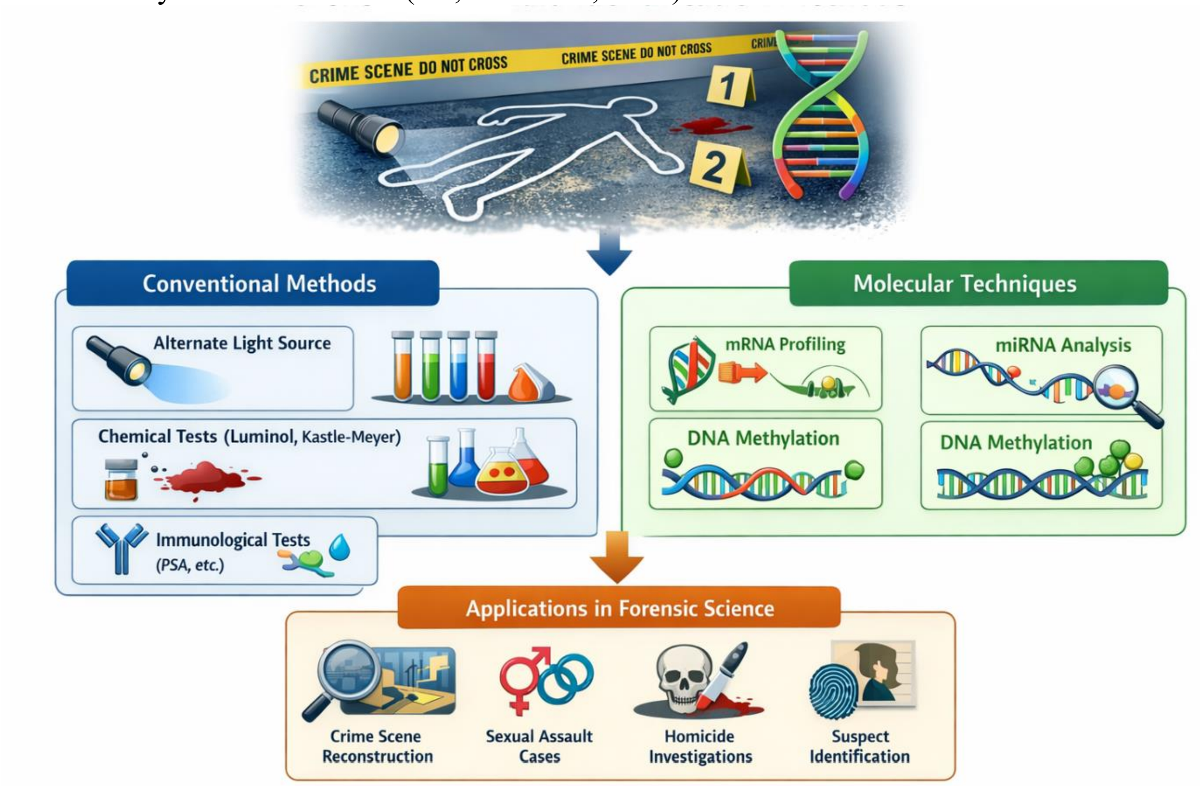

Figure.2: Basic & advance techniques to investigate at crime scene

An et al. and Zubakov et al. (2010) improved on earlier methods by creating the DNA Source Identifier (DSI)-Semen™ kit which is meant to replace the traditional way of looking at sperm cells under a microscope to identify semen in casework samples. This test uses MSRE-PCR to find DNA methylation patterns that are unique to semen at five different genomic loci. The findings showed that the DSI-Semen™ kit is strong and dependable able to find semen with only few of template DNA (Qu et al., 2021). Lee et al. investigated the utilization of tissue-specific differentially methylated regions (tDMRs) for forensic body fluid identification through bisulfite sequencing. This method finds out the DNA methylation status by looking for changes in nucleotides caused by sodium bisulfite treatment. During treatment methylated cytosines stay the same but unmethylated cytosines in CpG sites turn into uracil which is then read as thymine during PCR amplification (Roeder et al., 2022). This method was used to find the methylation profiles of five tDMRs by looking at combined DNA samples from blood saliva, semen, menstrual blood and vaginal fluid. This is a very precise way to tell the difference between different body fluids in forensic investigations.

Samples tDMRs linked to DACT1 and USP49 as markers that are only found in semen because they show hypomethylation patterns that are only found in this body fluid. In the same way, the PFN3 tDMR was suggested as a way to tell vaginal fluid apart (An & Zubakov, 2010). Recent studies have examined age related methylation alterations in semen specific tDMRs acknowledging that DNA methylation patterns can be affected by age utilizing samples from both younger and older males (Sakurada et al., 2024). Following the validation of the stability of these body fluid specific methylation profiles two multiplex systems were established to evaluate the methylation status of USP4, DACT1, PRMT2 and PFN3 tDMRs. These multiplex assays utilized both MSRE-PCR and methylation methodologies. Validation with 144 DNA samples showed that both systems worked to find semen with spermatozoa and to tell the difference between menstrual blood and vaginal fluid and other body fluids. This shows that they are reliable and useful in forensic science.

Chemical Techniques

The luminol test is a common way to find blood stains especially when the person who did it tried to clean up the evidence so it couldn't be seen by the naked eye. This method depends on the chemiluminescent reaction of luminol which gives off a blue-green light when it oxidizes. Hemoglobin and its derivatives contain iron which speeds up this process and makes it possible to find even small amounts of blood (Sijerčić Eberle et al., 2021). Luminol on the other hand is known to cause false positive reactions with a number of things, such as compounds that contain copper some bleaches saliva and a wide range of animal and plant proteins. The chemical reaction can sometimes damage DNA which could affect genetic tests that come after it (Sofer et al., 2019). Bluestar Forensic is another option. It is a bioluminescent reagent that is better at finding latent blood and is more stable. It is important that it does not damage DNA which keeps samples safe for later genotyping and forensic analysis (Zhang et al., 2023). Catalytic methods in forensic science are based on enzymatic reactions in which specific substrates undergo chemical transformation producing visible colour changes that facilitate the detection of biological materials as result (Coble & Butler, 2010). Numerous catalytic assays have been devised for the possible identification of blood predominantly utilizing the peroxidase like activity of the hem moiety in hemoglobin (Devaney et al., 2016). The heme group has intrinsic peroxidase like properties that allow it to break down hydrogen peroxide. This reaction creates reactive oxidizing species that then react with chromogenic substrates to cause colour changes that could mean blood is present (An et al., 2012). Benzidine and its derivatives ortho-tolidine & leucomalachite green, leucocrystal violet and phenolphthalein are some of the most common substrates used in these tests. The Kastle–Meyer test which is very popular is based on phenolphthalein (Forat et al., 2015). The benzidine test has been one of the most commonly used methods in the past. When blood is present it turns blue. This test is very sensitive but it has some problems especially the fact that it can give false positive results. Such inaccuracies may occur due to the presence of chemical oxidizing agents or naturally occurring peroxidases in specific fruits and vegetables thereby compromising the reliability of the test in forensic investigations (Forensic Science Regulator, 2020).

Because benzidine is known to cause cancer it is no longer used in forensic applications. Instead of this safer alternatives such as phenolphthalein-based assays are used. The Kastle–Meyer test which uses phenolphthalein and hydrogen peroxide together is now one of the most common tests for blood detection. In this method a swab or filter paper is usually used to collect a stain, and then phenolphthalin reagent and hydrogen peroxide are added one after the other. When phenolphthalin is oxidized to phenolphthalein, the reaction makes a pink color that is unique to this reaction. The Kastle–Meyer test is very sensitive and can find blood that has been diluted up to 1:10000. But like other catalytic assays it can give false-positive results because of chemical oxidizing agents and naturally occurring peroxidases in plant materials. Even with these problems one big benefit of this method is that it does not damage the sample, so it can be kept for later confirmatory tests, such as DNA profiling (Frumkin et al., 2008). The acid phosphatase (AP) test is one of the most common ways to find semen. The prostate gland makes an enzyme called acid phosphatase which is found in very high amounts in seminal fluid (Fulmer & Eden Green, 2018). The enzymatic activity of AP in semen is about 500 to 1000 times higher than in other body fluid, which makes it a good biochemical marker. This enzyme catalyzes the hydrolysis of phosphate esters producing products that subsequently react with specific colour developing reagents (Gandotra, Budowle & Chakraborty., 2020). When alpha naphthyl acid phosphate and Brentamine Fast Blue are present a dark purplecolor appears which could mean that semen is present (Gettings K.B. et al., 2015). Even though a lot of people use the acid phosphatase (AP) test is still very convincing because this enzyme can also be found in vaginal secretions and some other body fluids which makes it less specific (Goray et al., 2010).

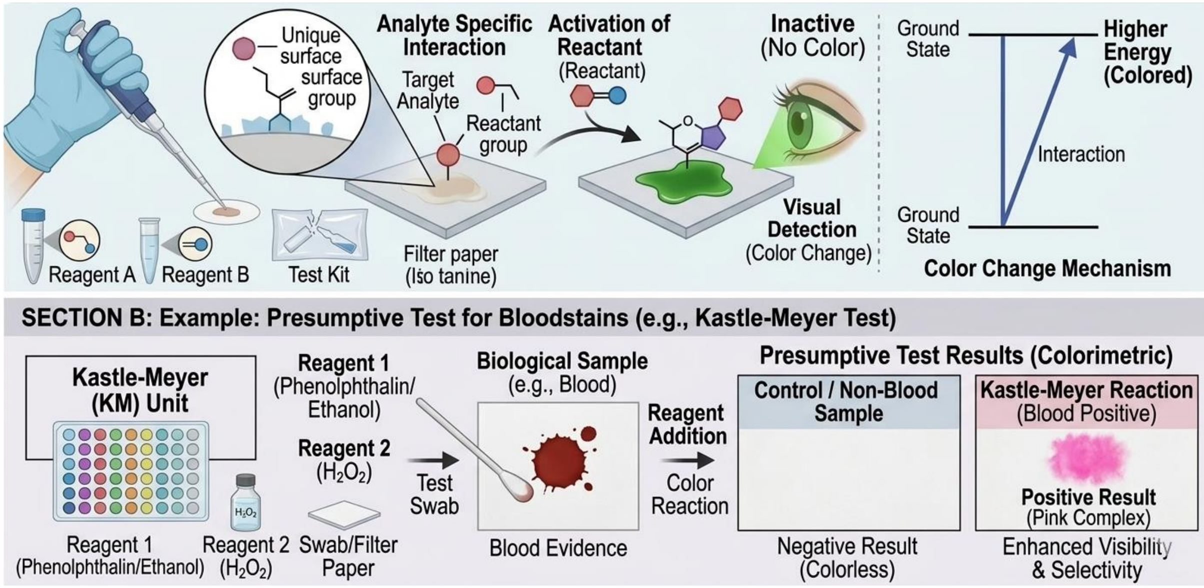

Figure.3: Chemical / Analytical methods used in forensics

UV light Sources

Using alternate light sources (ALS) like ultraviolet light, is a useful and non-invasive way to find body fluid stains that are hard to see with the naked eye (Steadman et al., 2020). Forensic scientists have made a number of commercial ALS devices especially for finding hidden semen stains at crime scenes. Wood's Lamp (WL) is a safe and easy-to-use device that gives off light in the 320–400 nm range. But it isn't very specific which means that other body fluids are more likely to give false positives (Tobe et al., 2019). Other devices are more sensitive and can do more things. The Bluemaxx™ BM 500 was able to find semen stains with 100% accuracy (An et al., 2012) and the Polilight® system can find many body fluids, including semen (Van den Berge et al., 2016). The newer Lumatec® Superlight 400 can see stains in both light and dark conditions and emits light over a wider range (320–700 nm). The best range for detecting semen was between 415 and 490 nm, and saliva was found in about 60% of the samples. Even though these are good things, the device didn't work as well on dark-colored or previously washed fabrics. Earlier reports said it worked the same on all types of fabrics (Zhang & Li, 2021; Weis et al., 2022).

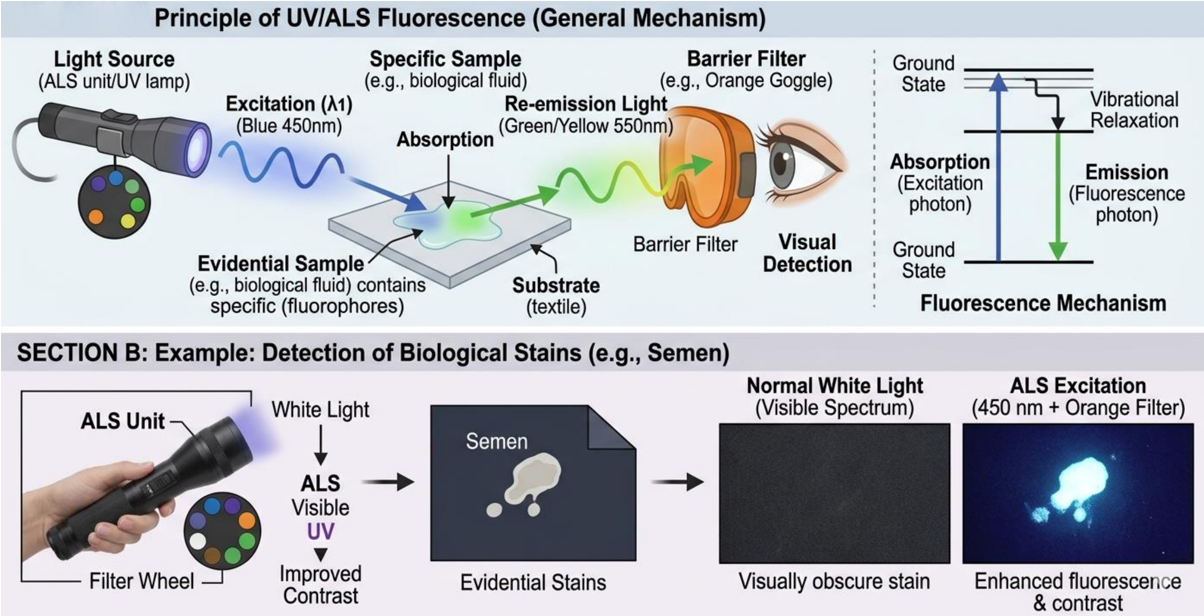

Figure.4: UV / ALS (Forensic Physics) methods used in forensic investigation

Discussion

In the last few decades there has been a lot of progress in figuring out how to find biological fluids in forensic investigations. For a long time forensic scientists used chemical & enzymatic tests to find & identify biological evidence at crime scenes. The Kastle–Meyer Test, Luminol Test & acid phosphatase assays are some of the most common traditional methods because they are easy to use can find things quickly & don't cost much. These methods are especially helpful at the beginning of forensic analysis when investigators need to quickly find biological stains that may not be visible to the naked eye. These presumptive tests frequently exhibit limitations including false positive results induced by substances such as plant peroxidases cleaning agents, or other environmental contaminants. Even though these methods are still useful for screening they need to be followed by tests that confirm the results to make sure that forensic evidence is reliable.

Immunological techniques have significantly enhanced the identification of specific bodily fluids. These methods depend on interactions between antigens, antibodies & they can find proteins that are unique to certain biological materials. Finding semen often means finding Prostate-Specific Antigen a protein made by the prostate gl&. Immuno-chromatographic tests have made forensic analysis more accurate faster & many forensic labs now use them all the time. Immunological tests can be affected by things like sample degradation cross-reactivity with similar proteins & low levels of target antigens in old samples. (An, J. H et al., 2012).

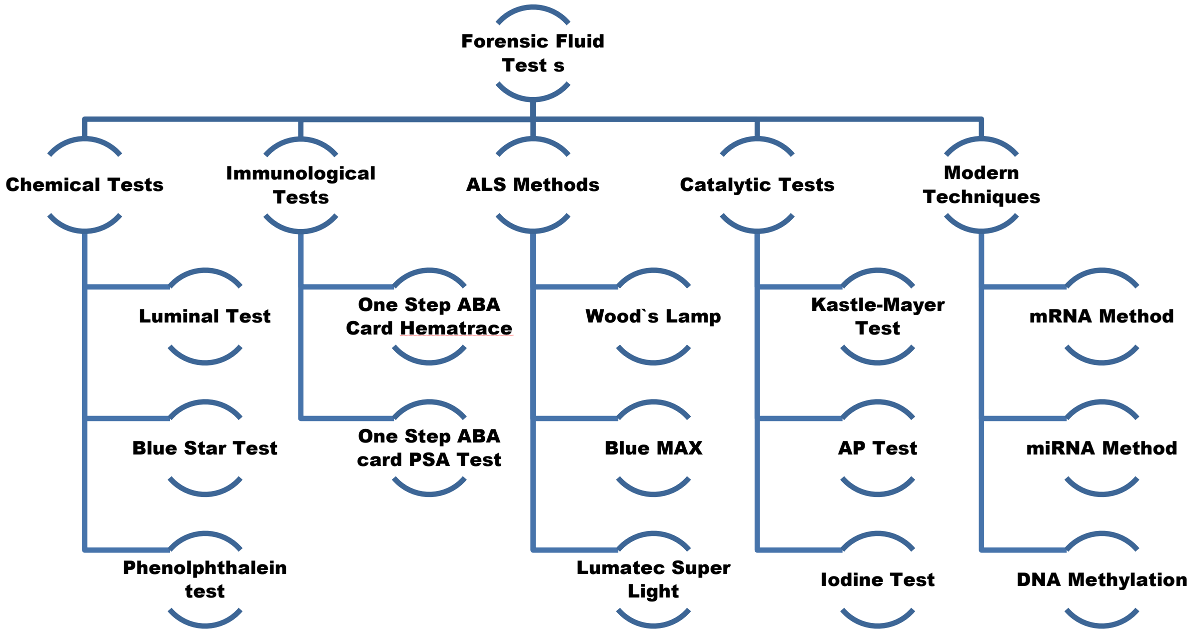

Figure.5: Running forensic Techniques

Molecular biology techniques have recently become very useful for finding body fluids. These methods depend on finding nucleic acid markers that show expression patterns that are specific to certain tissues. Messenger RNA profiling has garnered significant attention among these techniques due to its capacity to distinguish between various body fluids concurrently. Using reverse transcription polymerase chain reaction it is possible to find specific gene transcripts in blood & semen, saliva, or vaginal secretions. This lets you accurately figure out where a sample came from. One of the best things about mRNA analysis is that you can get RNA & DNA from the same sample. This lets you identify body fluids & profile an individual's DNA from the same piece of evidence. (Bauer, M. 2007). Using microRNA molecules as biomarkers is another important step forward in forensic molecular biology. MicroRNAs are tiny RNA molecules that don't code for proteins but control gene expression & are very specific to certain tissues. MicroRNAs are shorter and less likely to break down in the environment than mRNA molecules. This makes them especially good for analyzing old or damaged forensic samples. Research has shown that certain patterns of microRNA expression can reliably tell different body fluids apart. This shows how useful they could be in forensic investigations. Epigenetic methodologies have been investigated for the identification of biological materials. DNA methylation analysis has become a potent method for distinguishing tissues by their distinct methylation signatures. DNA methylation markers are stable and you can use the same DNA you used for genetic profiling to look at them. Methylation-based identification is a good choice for routine forensic work because it works well with current forensic DNA analysis workflows. But before these methods can be used in most forensic labs, they need to be tested and standardized more. Molecular techniques have a lot of promise, there are still some problems that need to be solved. Heat, humidity, ultraviolet radiation, & microbial activity are some environmental factors that can break down biological molecules & make forensic analysis less reliable. Also, molecular markers can be harder when they are mixed with other biological fluids, which is common in forensic casework. To make body fluid identification more accurate and reliable, it will be necessary to create multiplex assays & advanced analytical methods. In general, combining traditional biochemical tests with modern molecular methods is a full strategy for identifying forensic body fluids. Traditional methods are still important for finding biological evidence at first, but molecular techniques are better for confirming it because they are more specific & sensitive. Ongoing research and technological progress are anticipated to augment the capabilities of forensic biology & refine the interpretation of biological evidence in criminal investigations.

Conclusion

Identifying biological fluids is a key part of forensic biology & is very important for criminal investigations. Forensic scientists can put together crime scenes figure out what happened in what order & find links between suspects, victims, & places if they can accurately figure out where biological evidence came from. Forensic labs have always used chemical, enzymatic and immunological methods to find & confirm body fluids like blood semen, saliva, & vaginal secretions. The Kastle–Meyer Test Luminol Test, & immunological assays that look for Prostate-Specific Antigen are all popular because they are easy to use & give quick results. But these traditional methods often have problems such as lower specificity, the possibility of cross-reactivity with non-biological substances & lower reliability when working with degraded or trace samples. Recent advancements in molecular biology have markedly enhanced the precision & dependability of body fluid identification. The emergence of nucleic acid-based methodologies including Messenger RNA profiling, MicroRNA analysis, & DNA Methylation profiling, has yielded highly specific biomarkers that can differentiate various biological tissues. These molecular methods have a number of benefits such as being more sensitive being able to analysis degraded samples & working with DNA profiling methods that are used to identify people. RNA and DNA techniques are now more useful tools in modern forensic investigations.

Even with these improvements there are still some problems. Biological markers can become unstable because of changes in temperature, exposure to UV light contamination by microbes & long-term degradation of samples. The use of advanced molecular techniques necessitates specialised laboratory infrastructure st&ardised protocols & trained personnel. Consequently, additional research is essential to authenticate novel biomarkers, enhance analytical methodologies & create economical approaches suitable for forensic investigations. Future advancements in forensic biology are anticipated to incorporate novel technologies, including high-throughput sequencing sophisticated bioinformatics & epigenetic profiling to improve the identification of biological materials. Using both traditional screening methods & new molecular techniques together will probably make forensic body fluid identification more complete and reliable. Ongoing advancements in this domain will enhance the evidentiary significance of biological samples facilitate more precise & effective forensic investigations.

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