Developing a Method for Fluorescent Antibody Tagging for Identification of Female Cells in Mixed Forensic Samples

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Abstract

In the subject of forensic science and crime scene investigation, DNA has become more valuable than ever in providing crucial information for investigators**.** As the number of wrongful convictions decreases and the number of exonerations increases, DNA testing is the answer to accurately solving crimes. The purpose of this experiment was to study whether or not fluorescent tagging would be an effective method of identifying and separating male and female cells. It sought to determine if immunofluorescence can be applied to forensic science and technology. Rather than spending time sorting through the victim's DNA in order to get to the perpetrator's DNA, the suspect could be identified quickly and the case would come to a close. In this study, each cell count showed fluorescence, but most did not show enough fluorescence to meet the 75% threshold set for efficacy. The ideal forensic science identification method is one that is quick and easy to use in the field and eliminates error as much as possible. If an effective method of fluorescently tagging female cells for the purpose of separating genetic evidence is developed and modified as necessary, it could be the key to solving crimes where there is minimal evidence beyond the DNA samples.

Introduction

In the subject of forensic science and crime scene investigation, DNA has become more valuable than ever in providing crucial information for investigators (Harbison and Fleming, 2016). The Federal Bureau of Investigation first used it in 1988, and since then, it has become one of the most powerful methods of perpetrator identification (McKusuck et al., 1992). Because of the unique differences between each person's DNA, it is beneficial for scientists to have a method of identifying those related to the crime scene. DNA analysis is also one of the quickest methods of crime scene investigation (Romeika and Yan, 2013). Time and accuracy are key to solving crimes, particularly murders and kidnappings; giving the victims' families closure is the ultimate goal. Previous forensic body fluid identification techniques involved the use of chemicals and enzymes found in limited DNA samples (Harbison and Fleming, 2016). Tests such as these were not fine-tuned and often resulted in wasting of critical DNA materials. DNA profiling techniques must be gentle and preserve as much of the DNA sample as possible. As the DNA tests improve, they are focusing on limiting destruction of samples, as well as improving the time and effort required to perform each test.

The legal system is not perfect, and many convicted criminals have been wrongfully jailed. The United States has over 2 million incarcerated persons, the highest amount in the world (Ferner, 2016). Of those incarcerated, it is estimated that 3 percent are truly innocent. 149 people were exonerated in 2015, and the number of people exonerate each year is continuing to rise steadily. Studies have shown that the main cause of wrongful conviction is due to mistakes in the forensic testing techniques used for identification of criminals. Improvements in and further research into the techniques used by forensic analysts would significantly lower the number of wrongful convictions each year. Likewise, thousands of people have been exonerated

because of DNA testing many years after the original conviction. For one Louisiana man, testing of a hair sample nearly 30 years after the conviction exonerated him and cleared him of a crime that he did not commit (Registration of Exonerations, 2018). As the number of wrongful convictions decreases and the number of exonerations increases, DNA testing is the answer to accurately solving crimes.

Historically, the most common forensic science identification methods were chemical-based and often led to the destruction of cell material. These tests also were only truly beneficial as the first step in a 2-step identification process. When this type of forensic characterization began, there was approximately a 2% chance that there would be an error and an innocent person would be convicted (McKusuck et al., 1992). Because of their stability in forensic samples, RNA and DNA molecules are becoming more prevalent as an analytical tool. These methods maintain the cell structures and waste less of the biological material, and they also greatly reduce the chance of error in forensic identification. The future of forensic analysis lies in microbiological techniques because of their sensitivity and relative ease. However, they can be difficult to utilize because of the challenge of choosing the right markers for identification. A recent, and frequently studied and utilized, technique in forensic science analysis is the polymerase chain reaction (PCR) technique. This process imitates the natural copying of millions of pieces of DNA for the purpose of amplification and later identification (yourgenome.org, 2016).

Long non-coding RNA molecules serve the purpose of directing cellular processes (Raj et al., 2008). One particular molecule, Xist, serves the purpose of X-chromosome inactivation in female cells. This RNA molecule tells the cell to restrict one X-chromosome down to a Barr body. These molecules are unique to female cells, and the inactivation is random (Cerase, 2015).

Because Xist occurs only in female cells, a visualization of the molecules provides confirmation of the sex of the sample's original donor. Often in forensic science and crime scene investigation, investigators are met with a mixed cell sample of male and female cells, particularly in cases involving sexual assault (McKusuck et al., 1992). It is crucial for scientists to be able to identify the DNA and the suspect based on a DNA extraction. Before scientists analyze the DNA, knowing which cells need to be analyzed would save time and money. Knowing which cells contain female DNA and which ones contain male DNA would be beneficial.

Fluorescent in situ hybridization (FISH) is the primary method of identifying the location of an RNA molecule within a cell (Yue et al. 2014). Using immunofluorescence, specific bodies within a cell can be identified and located. This method had been used with specific probes for Xist as well as other types of RNA probes in living cells (Dean et al., 2015). Applying the FISH technique to forensic science requires modifying the protocol as necessary to work for dead cells from mixed samples.

This study was originally developed as a precursor to Virginia Commonwealth University's research with optical tweezers. If cells could be sorted based on male versus female cells, the cells could be arranged and the DNA could be extracted in order to separate the victim's DNA from that of the perpetrator. Rather than spending time sorting through the victim's DNA in order to get to the perpetrator's DNA, the suspect could be identified quickly and the case would come to a close. In the field of crime-solving efficiency is key, and a method to improve the efficiency of DNA analysis would improve the success rates of police departments in closing cases.

Developing a Method for Fluorescent Antibody Tagging for Identification of Female Cells in Mixed Forensic Samples

The purpose of this experiment was to study whether or not fluorescent tagging would be an effective method of identifying and separating male and female cells. It sought to determine if immunofluorescence can be applied to forensic science and technology. A FISH method was used to determine the location of the Xist RNA molecule in female epithelial cells. Using the Affymetrix QuantiGene® ViewRNA ISH Cell Assay Kit, Xist molecules were identified within cells. The method was idealized for dead female epithelial cells and then tested. In each view of each sample the number of cells demonstrating immunofluorescence were compared with the cells not demonstrating immunofluorescence to determine the percent efficacy of the technique.

The independent variable for this experiment was sample, section, and view; the dependant variable was the percent of cells demonstrating fluorescence. Constants were the materials used, the subject the samples came from, the testing environment, and the methods used for testing. The null hypothesis (H_0) for this experiment stated that if 75% or fewer of the dead female cells showed fluorescence, then fluorescent tagging was ineffective. The alternate hypothesis (H_a) for this experiment stated that if more than 75% of the dead female cells showed fluorescence, then fluorescent tagging was effective.

Materials and Methods

For this experiment, the Affymetrix QuantiGene® ViewRNA ISH Cell Assay Kit was purchased from ThermoFisher. The kit contained all of the necessary reagents for the amplification and detection of microRNAs.

Safety gear was worn throughout the entire protocol. Special caution was taken with the reagents labeled as mutagens. First, the bench was disinfected with bleach and ethanol to break down any RNA molecules. 2 buccal swabs were obtained and the cells were released. Each

fresh swab cutting was placed into a spin basket and centrifuged for 5 minutes at 5,000 rpm to dry. The swabs were removed and placed into a tube with 1X phosphate-buffered saline (PBS). By flick-mixing for approximately 5 minutes, the cells were released into solution. The swabs were removed by using sterilized forceps.

With the suspended cells, the protocol continues with Appendix A: Sample Preparation Procedure for Suspension Cells ("QuantiGene ViewRNA ISH Cell Assay User Manual", 2011). The cells were fixed in 4% formaldehyde solution, which was prepared under a fume hood, and then secured to poly-L-lysine coated microscope slides with two hydrophopic barriers. A portion of cells from one swab was added to each of 2 barriers. Two more sections were created on a second slide with the second swab. The slides were then stored in 100% ethanol until they were used again.

In preparation for the assay, an incubator was prepared at 40 °C and reagents were thawed, placed on ice, or warmed according to what the protocol specified. The cells were then rehydrated using ethanol and 1X PBS and then permeabilized in a detergent solution. After that, the cells were digested with a protease solution. The cells were then hybridized with the probe sets. ViewRNA Homo sapiens XIST (RUO) Probe was used. Subsequently, the cells were then hybridized with pre-amplifiers, amplifiers, and label probes according to the protocol. Once hybridized, the cells were then stained with a 4',6-diamidino-2-phenylindole (DAPI) solution and visualized under a fluorescent microscope.

Each cell sample had two sections, and each section was visualized in 5 counts. In each of the 20 total counts, the number of cells demonstrating immunofluorescence were counted as well as the total number of cells. The percentage of cells fluorescing was calculated in order to determine the efficacy of the method.

Results

In this study, each cell count showed fluorescence, but most did not show enough fluorescence to meet the 75% threshold set for efficacy. Only in counts 13 and 16 was the fluorescent count high enough to meet the threshold. On average, 3.3 cells of each count showed fluorescence, unlike the mean number of cells of each count that needed to show fluorescence in order to meet the 75% threshold which was 5.4 cells. A t-test was run on the data and it yielded a p-value<0.05. This result means that the difference between the experiment results and the 75% threshold was significantly different.

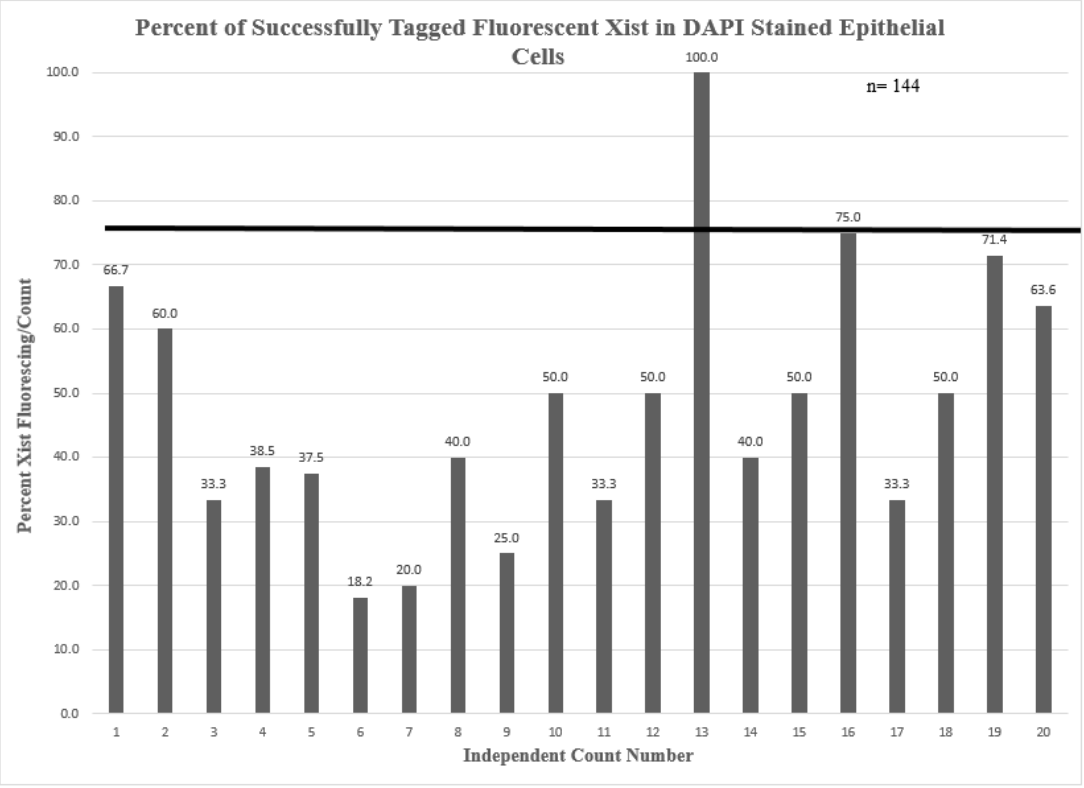

Figure 1. Percent of successfully tagged fluorescent Xist in cell counts. The percentage of cells fluorescing was only achieved in two counts, number 13 and 16. In the majority of counts, the percent of fluorescing Xist cells was not enough to meet the 75% threshold. A t-test was run on the data, and p<0.05.

Conclusion

The percentage of cells showing fluorescence in each count was statistically different from the 75% threshold. After conducting a t-test on the data, a $p<0.05$ was attained. Thus, the null hypothesis cannot be rejected *H_0: If 75% or fewer of the dead female cells showed fluorescence, then fluorescent tagging was ineffective*.

The low p-value indicated that the data were not statistically similar to the 75% threshold for determining efficacy. This result was obtained for multiple reasons. For example, the protocol used in this experiment is a long protocol that had never been used in this context before. Before testing could even begin, the protocol had to be optimized, which left little time for the cell testing. Further, the average percentage of fluorescence increased between samples, therefore it is possible that the protocol would be effective if it was thoroughly practiced and optimized, providing more reproducible counts.

The large statistical difference between the cell counts and the 75% threshold could also have been due to poor cell adhesion. When the protocol was still being optimized, a recurring problem was cell retention throughout the protocol. An alternate adhesion protocol was provided and was utilized, which provided better results, but the retention was still not as good as hoped for. Due to the length of the protocol and the types of reagents used, it is possible that this protocol does not result in a large percentage of cells retained, and thus a low percentage of fluorescence within the cells.

One improvement of this experiment would be to utilize a protocol that requires even less time to complete. In previous studies, protocols of fewer than 5 hours have been used, and they have yielded successful results (Yue et al., 2014). In a field where time is crucial, quick and effective protocols are necessary. In this experiment, the protocol required approximately 9

hours to complete. Unfortunately, in crime labs, a 9-hour protocol is not useful because of the amount of time that is required to produce results. Additionally, if a shorter protocol were utilized, but for the same hypothesis, it is possible that more data could be collected in a shorter timeframe, and the experiment may yield better results.

The ideal forensic science identification method is one that is quick and easy to use in the field and eliminates error as much as possible (McKusuck et al., 1992). This perfect technique would also utilize a marker that is highly distinguishable among samples. An ideal method would also use non-coding RNA molecules to avoid confusion with behavioral characteristics and diseases. The idea of a "perfect" forensic identification method served as the basis for this project. This protocol utilized Xist, a long, non-coding RNA molecule, as the tag and marker of identification in order to separate male and female cells. Xist is only found in female cells, and thus it would be a clear way to distinguish the female cells of a victim from the male cells of a possible perpetrator, or vice versa. Fluorescent tagging of Xist could be the answer to solving crimes potentially involving a male and a female because of the clarity and differences shown between cells in each sample.

In the winter of 1964, 19-year-old Mary Sullivan was raped and strangled to death (Bulman, 2014); she was one of 11 women killed by the "Boston Strangler." Several years after, Albert DeSalvo was convicted and sent to prison on multiple rape charges. Nearly 30 years later, after DeSalvo claimed to be the "Boston Strangler," DNA evidence linked Sullivan to DeSalvo. In 2013, Boston Police Department reopened the case and performed familial testing using DNA from DeSalvo's nephew and compared it to seminal fluid found on Sullivan's body. Despite the fact that both Sullivan and DeSalvo were deceased, Sullivan's family was able to get closure

knowing the truth about her death. This is just one of numerous cold cases that have been solved using DNA testing.

If an effective method of fluorescently tagging female cells for the purpose of separating genetic evidence is developed and modified as necessary, it could be the key to solving crimes where there is minimal evidence beyond the DNA samples. Once a protocol is developed, it would be implemented in crime labs and technicians would be trained with the intent of replacing other techniques like fingerprint analysis with DNA analysis, and FISH specifically. As new research emerges about how much more accurate DNA is in proving guilt compared to the fingerprint analysis of the past, it is likely that the use of DNA in forensic science will become even more prevalent (Harbison and Fleming, 2016). Forensic science and crime scene investigation need to be as fool-proof as possible in order to prevent wrongful convictions. When there are lives on the line, there is no room for error. A more effective technique must be developed in order to improve the efficiency and accuracy of DNA analysis, which is the future of forensic science.

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