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hybridization of the probe to DNA other than the target. Endogenous biotin may be detected in cells even with the use of endogenous biotin blocking procedures. A simple solution to nonspecific staining caused by nonspecific biotin detection is to replace biotin with a hapten that does not occur naturally within human cells. Nonendogenous haptens are compatible with TSA-FISH techniques and should not result in nonspecific detection events. Nonspecific hybridization of the probe to DNA other than the target sequence can be alleviated through a variety of approaches. It is important to ensure that the stringency of the hybridization is high enough to reduce nonspecific hybridization events, but low enough to allow the probe to hybridize to the target sequence. Similarly, the post hybridization wash conditions should be stringent enough to remove loosely bound or unbound probes, but not so stringent that specifically hybridized probes are removed. Optimum stringency conditions should be determined empirically. Typically, nonspecific hybridization should not be addressed by increasing the length of the probes. First, a minimum of 18 bp should be sufficient to create a probe specific to a desired DNA target under optimum hybridization and wash conditions. Second, FISH probes are subject to rigid size parameters dependent on the type and purpose of the probe. It is not recommended to increase the probe length in most instances.

Although this research was not able to produce a successful FISH assay for discriminating the ABO blood groups, important techniques were developed and optimized for FISH LM, which can be applied to the separation of mixtures of male and female cells that have the same cell morphology. Methods were optimized to reduce the amount of background fluorescence. When samples fixed to polyethylene naphthalate (PEN) and polyethylene teraphthalate (PET) membrane slides were subjected to standard

FISH testing with Vysis CEP X® and Y® alpha satellite probes, it was observed that PEN slides are highly auto fluorescent whereas the PET slides demonstrate little to no auto fluorescence. Increased background signal caused by auto fluorescence may interfere with fluorescent probe signal interpretation. These results indicated that PET slides, not PEN slides, should be incorporated into fluorescent LM procedures. Furthermore, the research demonstrated that pretreatment of PET slides with pepsin buffer, followed by post-fixation of the cells in buffered formalin can significantly reduce the amount of background fluorescence thereby increasing the sensitivity of the probe system.

Working protocols for FISH LM were developed for techniques not currently used in the forensic field. The techniques that were developed for the hapten labeling of FISH probes using biotin may prove beneficial in future LM research. With further optimization, tyramide signal amplification (TSA) may be a valuable technique in forensic applications of fluorescent microscopy. While the first attempt to adapt these techniques for forensic use was unsuccessful to differentiate ABO blood groups, the basic techniques of TSA were developed and can be used to increase the signal intensity and sensitivity of FISH probes associated with genetic marker systems being developed for future forensic research.

Implications for Policy and Practice

At this time, these findings have no direct impact on policy or practice. The research indicated that the FISH probe method could not be used to separate mixtures of cells from individuals with different ABO blood groups. Based on this research, FISH laser microdissection cannot be applied to separating cellular mixtures that contain the

same cell morphology and same gender. However, previous research has shown that laser microdissection techniques can be used to separate forensic mixtures consisting of cells that differ in morphology, gender, and the separation of biological cells from soil or other particulate matter (6,7,8,9,27,28).

The research performed has indicated that there are FISH techniques, such as tyramide signal amplification, that previously have not been explored for forensic applications. Many of these techniques have been utilized for years in clinical and diagnostic applications. Consideration of alternative FISH methods could expand forensic LM-FISH capabilities beyond standard X and Y chromosome FISH probing, thus expanding the forensic uses of laser microdissection to separate mixtures of cells from evidentiary samples.

Implications for Further Research

This research has laid the groundwork for NIJ award number 2009-DN-BX-K250, "Identification and Separation of Evidence Mixtures Using SNP-Based FISH Techniques and Laser Microdissection." NIJ award number 2009-DN-BX-K250 proposed the development of a fluorescent probe screening system based upon human genetic single nucleotide polymorphisms (SNPs) that could provide a basis for the separation of mixture samples with LM technology. Based on ABO probe research, alternative types of FISH probes will be examined to determine if any might be suitable for this level of discrimination. Also, the probes will be labeled with haptens other than biotin to eliminate the effects of nonspecific biotin detection. Other haptens that are commonly used with TSA-FISH procedures include digoxigenin and dinitrophenol. To address issues related to insufficient biotinylation of the probes, probes for NIJ award number

2009-DN-BX-K250 will be chemically synthesized with a specified amount of digoxigenin or dinitrophenol incorporated by a commercial manufacturer.

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Dissemination of Research Findings

The NIJ Conference 2009, Washington, D.C., July, 2009

- Poster Presentation: "Physical Separation and STR Analysis of Forensic Mixtures Using Laser Microdissection and Fluorescence In Situ Hybridization" R. Driscoll, H. Cunningham and R. Bever

19th International Symposium on Human Identification, Madison, WI., October, 2008.

- Poster Presentation: "Physical Separation and STR Analysis of Forensic Mixtures Using Laser Microdissection and Fluorescence In Situ Hybridization" R. Driscoll, H. Cunningham and R. Bever