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Document Title:	**DNA Forensics Using Single Molecule Technology: From DNA Recovery and Extraction to Genotyping Degraded and Trace Evidence without PCR**
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Document Number:	**251812**
Date Received:	**July 2018**
Award Number:	**2011-DN-BX-K542**

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Final Technical Report

Title: DNA forensics using single molecule technology: from DNA recovery and extraction to genotyping degraded and trace evidence without PCR

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Abstract

PCR based DNA profiling technology is the gold standard for human identification technology, being very discriminatory and requiring only several cells worth of DNA. However it is still useful to consider alternative technologies which fundamentally re-imagine how forensic DNA evidence is characterized and typed. Wholly new approaches may provide supplemental information under particularly challenging conditions, for example with low copy numbers samples, mixtures, and damaged DNA (eg. abasic sites or fragmented loci). Alternatively, novel approaches may be developed that don't require the time or training associated with current typing methods, making DNA profiling more economical and broadly available.

One concept is to apply contemporary single molecule techniques to forensic analysis, with the goal of being able to interrogate DNA samples one molecule at a time, measuring each molecule repeatedly to guarantee confidence in the result. The single strand is the ultimate LCN sample. Furthermore, single strand profiling will lead to single cell genotyping, allowing mixtures to be conclusively resolved in those cases where the sample can be typed cell-by-cell. Finally, because the approaches investigated here do not require chemical amplification, we also consider their utility in investigating damaged DNA.

In this basic research, we develop proof of concept experiments which demonstrate the potential application of single molecule techniques to forensic samples. We first demonstrate that the forensic loci in genomic DNA can be specifically targeted for covalent attachment to a surface. This step is essential because the weak signal from a single DNA strand must be recorded for several minutes in order to determine its identity, which requires it to remain at a fixed position during the measurement. The attachment is accomplished by coating a surface

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with hairpin structures designed to be ligated specifically to the forensic loci in a DNA-restriction enzyme digest. Covalent attachment not only localizes the DNA to one spot for prolonged measurements, it also permits the labeled primers and probe oligos to be melted off and rinsed away without losing the original DNA strand. This fact permits repeated measurements of the same DNA molecules, potentially improving reliability.

Second, we show that we can anneal tandem repeat DNA sequences to the STR region of the locus, and that by using fluorescently labeled repeats, it is possible to determine the size of the locus via single step photobleaching. Single stranded DNA samples are allowed to anneal with 8 bp repeats (two 4 bp repeats in tandem) which are labeled with Alexa 488 or gold beads. A ligase links the 8 bp repeats together as they anneal to the DNA, increasing stability. Illumination with a laser over several minutes results in the photo-destruction of the dyes, one by one, and the resulting stepwise drop in the fluorescence signal is used to determine the original number of dyes, and therefore repeats, at the locus. Alternatively, the samples labeled with gold beads are imaged in a TEM and the number of repeats determined by counting the beads.

In contrast to the surface attached studies, freely diffusing DNA samples were also characterized using fluorescence quenching. Primers containing fluorescent labels and gold bead quenchers were annealed to the DNA. Although the complex dependence of quenching on DNA length, bead size, and buffer made it impossible to quantitatively calculate the size of the locus, it was possible to use a look-up table generated by measuring control samples that included all possible lengths and bead sizes. These initial results used at most two bead/dyes. Comparing the results for the measured unknown to the look-up table resulted in correct identification more than half the time. This percentage is significant as a proof of concept work, but further development is needed to raise the success rate to a forensically acceptable level. The lack of amplification means no time spent thermally cycling, which offers the possibility of developing a quick protocol where a sample is extracted and measured directly.The potential for this technique to be applied to DNA with abasic sites is also discussed.

Finally, we examined the effectiveness of a pre-amplification protocol for PCR. We assumed that many of the difficulties encountered with chemical amplification are due to the fact that transcription errors in the early cycles are amplified along with the rest of the DNA. By using only a single primer in the preamplification cycle, copying rates are much lower, but only the original DNA is copied. After the preamplification, only copies of the original DNA was present, not copies of copies. Although the results were an improvement (in peak height and lower drop out) over the LCN techniques used when this research began, the reality is that the PCR approaches have advanced quite rapidly, outperforming this approach and without additional handling. We include the results here for completeness, as it was work explored by this funding, but unless a particular niche is found where it is peculiarly suitable, we don't anticipate continuing this work.

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Table of Contents

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Executive Summary

Introduction

PCR based forensic analysis has been enormously successful, with some commercial kits currently requiring just a few cells worth of DNA to obtain a profile. In fact, there is no intrinsic physical limitation in the low copy limit of DNA amplification. After all, cells *in vivo* routinely repair and transcribe single DNA molecules with high fidelity. Still, it is worth considering what ultimately may be achievable *in vitro*. Some questions worth considering are:

1) Will current techniques evolve to be able to reliably type a single strand of DNA?
2) In current PCR techniques the original DNA is not recovered. Can techniques be developed that allow the original strand to be recovered and repeatedly typed?
3) Even *in vivo*, cells are challenged by the accumulation of damage. What is the limit of information that can be extracted from samples containing damage such as abasic sites or fragmented loci?
4) Thermal cycling is time consuming. Can faster protocols or alternative techniques reduce the time requirement?

The first question addresses the ultimate low copy number (LCN) sample, but is also relevant to mixture samples containing a minor contributor whose DNA constitutes a small fraction of the total sample. Currently, even though chemical amplification of LCN samples is possible, the signal can be weak and the process itself may introduce stochastic and background signals. As a result, proper interpretation of the data can be difficult. Unless it is clear that further improvements in the biochemistry protocols will solve these problems, it is wise to explore other complimentary approaches.

Even if the current protocols are refined to produce clear results, there is still an issue with being able to trust the results. Errors and artifacts are always possible, especially for DNA which has been degraded by age, exposure, or the presence of impurities. Ideally, if the results come into question, one could simply retest the sample. However, current protocols do not allow for the original DNA sample to be recovered, and once an LCN sample is tested, it cannot be re-tested. Therefore, as a way to potentially circumvent these issues, it is advisable to seek novel approaches that are fundamentally different from PCR, approaches which do not consume the sample that is being tested.

For more than two decades now, single molecule research technology has been dramatically enhancing biological and biophysical investigations. As the name implies, the high sensitivity of the equipment can record the signal generated by individual molecules. The data is then analyzed to classify each molecule according to some criterion, such as structure, composition, or chemical characteristics. In the context of forensic analysis, this technology promises to be able to characterize each strand of DNA in a sample individually, and without PCR. This technology would be the ultimate in LCN analysis. One could then imagine resolving mixtures by genotyping individual cells. Furthermore, by avoiding the process of enzymatic amplification, the challenges involved in analyzing damaged DNA can be mitigated.

However, single molecule technologies are currently a basic science tool used under pristine conditions. Before such ambitious forensic applications can be realized, it is necessary to

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first identify single molecule technologies which are suitable for forensic investigations, and determine how real world forensic samples can be prepared for reliable single molecule genotyping.

In this report, we explore several different strategies for typing DNA without enzymatic amplification. We determine the suitability of these techniques for examining single molecules of DNA, and explore protocols for preparing genomic DNA for single molecule analysis. Our results indicate that there is indeed a potential for single molecule forensic analysis. In particular we show that it is possible to deposit genomic DNA in area sufficiently small to scan using single molecule technologies, immobilize the DNA to prevent its loss, and characterize the DNA repeatedly using single step photobleaching. Furthermore, these techniques can be non-destructive, allowing the DNA to be retained for later confirmatory testing, enzymatic amplification, or stored for analysis with future advanced techniques.

STATEMENT OF THE PROBLEM

DNA Genotyping in Forensics

DNA forensic techniques have matured to the point that minute quantities of DNA (~ 100 pg, or the equivalent of ~ 15 cells) are sufficient to reduce the number of possible contributors down to a few or a single individual.[1–3] One of the primary techniques is the identification of short tandem repeats (STR),[4] although interest in single nucleotide polymorphisms (SNP) and next generation sequencing has been growing rapidly. Short tandem repeats are stretches of DNA which have short nucleotide sequences (typically four base pairs) which repeat several times. The number of repeats varies from person to person but is constant in a particular individual (with rare exceptions). The number of repeats is therefore a marker which is used to characterize an individual. Using a number of different STR loci, the list of possible contributors of a DNA sample can be narrowed to only a few individuals. Currently 15 to 20+ loci are used, with more being investigated.

Genotyping such minute quantities of DNA is possible due to the use of polymerase chain reaction (PCR) amplification. PCR involves heating the DNA to melt the double strand into two single strands, each of which upon cooling is re-polymerized enzymatically into a double strand, doubling the amount of DNA in the sample. With repeated cycles, the amount of DNA increases exponentially, until sufficient quantities exist to characterize the size via capillary electrophoresis (CE). In addition to instrumental noise, enzymatic amplification can also produce signals which are the result of transcription errors being amplified in later cycles. In a typical sample from a single contributor, these signals are significantly lower than the true peaks of the DNA profile, which permits the setting of a threshold in order to distinguish STRs from other artifacts.[3,5] PCR amplification typically ensures that only the loci of interest are present in sufficient quantities to exceed these thresholds.

PCR Amplification Issues

Difficulties rarely occur in profiling samples as long as the amount of DNA evidence at start is above ~ 100 pg. However, sometimes the samples collected fall below this threshold,[6] and the sample is termed low copy number (LCN). The analysis of these samples can be problematic, first of all because the actual DNA present is subject to stochastic variations. In an LCN sample

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which averages 12 copies at each locus, it is possible that by chance one locus may have 19 copies present, while another locus has only 5 copies. Therefore peak imbalances are often present in LCN samples. Second, PCR amplification is not identically efficient from locus to locus and run to run. Some peaks may be amplified normally while others are diminished, adding to the imbalance in the signal. Third, signals are weak to begin with. Stochastic sampling and variations in amplification efficiency can mean that already weak signals (which should be present) may not be amplified sufficiently to be significantly above the background, resulting in drop-out.[6–9] At the same time, peaks originating from sources not associated with the contributor may drop-in. This drop-in of a new peak can be caused by contamination with another donor, or by the PCR enzyme shifting its position by one set of repeats, creating a stutter peak which can sometimes be larger than predicted, thereby falsifying the output.[10,11] Imbalances in the output occur due to the stochastic nature of polymerization and the fact that early fluctuations are subsequently amplified.[10] Collectively these issues mean that for LCN DNA samples, the results that are produced may be called into question.[6–9]

A separate but related problem in DNA analysis is samples which are degraded or contaminated to the point that PCR amplification is not possible.[15] Nicks and breaks in the DNA cause it to fragment upon melting in the PCR cycle. Once a locus is broken, it can no longer be amplified. Degraded DNA may also contain abasic sites where polymerization will stall, or contaminants which prevent re-polymerization. In any of these cases, attempts to PCR the sample fail.

Current Approaches to Problems

The importance of the issue has resulted in many variations in the collection,[7,12] amplification, analysis, and interpretation of degraded and low copy number samples. Different practices for collecting DNA include varying the swab material,[16,17] using wet or dry swabs and varying the number of swipes,[18,19] and trying different solvents.[16] During amplification it is possible to gain some improvement in the results by increasing the number of PCR cycles, thereby amplifying the DNA more.[6] For degraded DNA samples, results can be improved by moving the start and end of the PCR region closer to the repeat region, creating so-called mini-STRs,[20] making it less likely that breaks occur within the locus. There has also been some success in repairing DNA damage enzymatically by using ligases and polymerases.[21–26] Other attempts to improve amplification include a post-PCR purification/concentration step, improved fluorescent tags, whole-genome amplification,[27,28] or using additives which make the PCR more efficient.[29,30]

Another approach is to try to confirm results from challenging samples by splitting the sample and analyzing it twice or more.[6] It is unlikely that the same stochastic variation will occur twice, however splitting the sample increases the likelihood of stochastic effects in the first place, and in the case of disparate results it may not be possible to identify the "correct" outcome. Furthermore, by its nature LCN data does not lend itself to being split more than 2 or 3 times. Many of the variations in the protocol require validation studies to develop new analytical thresholds. The existing thresholds to separate signal from artifacts were established and validated under different experimental conditions.[5] Applying existing thresholds to new conditions is unreliable, and proper interpretation of the complex results from more challenging samples requires accurate modeling of the likely outcomes, including estimating the probability

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of drop-in, drop-out, and the variation in peak heights.

Ultimately, despite numerous attempts to optimize current procedures, obtaining reliable results from degraded or LCN samples remains challenging.

PURPOSE

Given the current difficulties in analyzing low copy number and degraded DNA samples, and the fact that such samples occur in high value crime scenes such as missing persons, national security incidents,[6,31] and human remains identification, there is a critical need for alternative technologies which can effectively and reliably characterize this evidence. Any new technology should fundamentally address the problems of low copy number and degraded DNA, and if possible be non-destructive in order to allow repeated testing of the limited material.

Single molecule techniques have been developed for biological and biophysical investigations over the last 15 – 20 years, with the techniques becoming more quantitative, robust, and diverse over the last 10 years. Under laboratory conditions, these techniques combine the high sensitivity, specificity, resolution, and discrimination necessary to identify and characterize individual molecules. Cleanliness in laboratory samples is essential for the techniques to work because the sensitivity of the equipment will detect even very small concentrations of impurities. Therefore, in order to apply this technology to forensic evidence, protocols need to be developed which remove contaminants that would provide false signals, localize the DNA within the field of view of the equipment, and allow for nondestructive testing which would facilitate repeatable measurements on the same molecule. If these goals can be met, the sensitivity to investigate single molecules suggests an ability to examine DNA evidence with no PCR amplification.

Not all single molecule technologies are expected to be easily adapted to forensic work. The purpose of this research is to explore different technologies and strategies for preparing forensic samples in order to identify those which are suitable for the forensic analysis of DNA.

RESEARCH DESIGN

The concept of using single molecule techniques to circumvent PCR is not entirely new. With an eye towards personalized medicine, single molecule approaches to gene sequencing is an area of active research.[32–37] Progress is also ongoing in attomole DNA detection[38] and fluorescently tagging double stranded DNA in a sequence specific manner.[39–42]

However, single molecule procedures are still largely a basic science tool, used in laboratories under pristine conditions with samples that have been contrived to fit the experimental needs. For example, to reduce unwanted noise, the field of view in a single molecule measurement is made as small as possible, typically not exceeding tens of micrometers. Rather than meticulously scan a surface looking for a single molecule with this tiny field of view, researches instead cover the surface with a low density of molecules, guaranteeing that no matter where they look, there will be a molecule in the field of view. It is common to use microliter amounts of samples at picomolar concentrations, but that still means a droplet containing approximately 10^{-18} moles, or tens of thousands of molecules, very much in excess of the LCN threshold.

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Furthermore, with so many molecules in the sample, and the samples relatively easy to generate, there is usually no need to develop protocols that ensure that the same molecule is interrogated repeatedly. Instead the research investigates many different molecules, one at a time. Ultimately, a forensic application of the technology will require an approach where the same molecule can be interrogated many times to increase the robustness of the results.

This project addresses two issues directly: when DNA molecules are rare, how can they be effectively trapped in the field of view of the equipment, and once localized, how can they be effectively characterized.

Introduction to Single Molecule Fluorescence

To better understand the challenges in applying single molecule technologies to forensics, an introduction to current single molecule imaging techniques is appropriate.

Molecules anchored to a surface are imaged with an intensified CCD camera at high magnification. Each pixel in the image corresponds to the diffraction limited resolution of approximately 500 nm on the surface, minimizing the volume from which background noise is collected. Background is additionally kept low by bringing in the excitation light at an angle shallow enough to guarantee total internal reflection at the glass/water interface. With total internal reflection, none of the excitation light actually enters the solvent and therefore very little scatter is generated. However any fluorophore near the surface will still undergo excitation due to an evanescent wave which penetrates about one wavelength into the solvent. The photons subsequently emitted by the fluorophore are imaged with a CCD camera. Attached molecules can be observed over a length of time (seconds or minutes or longer) and simultaneous imaging of many molecules scattered on the surface over hundreds of microns is possible. Imaging can continue as long as the dye remains fluorescent. Eventually, fluorescence is destroyed (by photobleaching the molecule, for example) and the experiment is over.

One widespread method for counting molecules is to observe single step photobleaching. The brightness of a fluorescence signal depends on the number of fluorophores present. When observed for long periods of time, molecules will eventually be photo-destroyed. As each molecule is destroyed, the fluorescence will drop a discrete amount. When the number of fluorophores is small (< 20), these discrete drops in fluorescence are visible as a single steps in the brightness of the sample. The fluorescence signal is recorded until all of the fluorophores have been destroyed and the sample is reduced to background brightness. The number of discrete steps recorded are then counted to determine the number of fluorophores originally present. This method is nearly universally used to confirm that single molecule observation are indeed single molecule (by the presence of only a single step to background), but the techniques has also been used to count molecules, such as nicotine acetylcholine receptors[43] or CFTR in plasma membranes.[44]

Immobilizing forensic DNA samples

In order to make measurements over seconds or minutes, molecules need to be trapped in the field of view of the camera. Furthermore, the sensitivity of the techniques requires the the field of view be free of contaminants. The ideal surface for such measurements is one which preferentially binds DNA above other contaminants and labels the forensic marker on the DNA

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so that it is unambiguously visible to the instruments.

In this project our goal was to develop a method to covalently link DNA forensic markers to a surface and them label them with a probe for interrogation. As a proof of concept, we use traditional single molecule techniques which cover the surface with molecules in order to be able to easily locate test molecules. Once the technique is established, a forensic application will require working with LCN DNA samples. We expect that such an adaptation will be less difficult that developing the attachment protocol itself. In the case that only a few DNA molecules are present, the attachment strategy need not be modified, but instead the camera must search to find the molecules on the surface. Since the field of view of a CCD camera is typically about 0.5 mm wide for a 1024 x 1024 pixel camera (at the highest diffraction limited resolution), the droplet area from a small volume samples of 20 – 30 µl can be searched with several CCD images.

Attachment of the DNA is accomplished by fragmenting the DNA using a restriction enzyme which cuts the DNA at a site near the TPOX forensic markers. The strategy is illustrated in Figure 1. The surface is prepared by coating it with a high density of specially designed DNA hairpin oligonucleotides which have three regions: a linker capable of covalently binding to a chemically reactive surface, a single stranded region that is complimentary to the DNA locus after digestion, and a hairpin loop with a stem that provides the double stranded region necessary for efficient ligation of the DNA locus to the oligonucleotide. After fragmentation, the DNA evidence is placed on the surface, and the fragments are allowed to anneal to the surface bound oligonucleotides. The sequence of the complementary region of the oligonucleotide is used to guarantee specificity and ensure that of all the sites cut by the restriction enzyme, only the site near the marker will anneal to the oligonucleotide. The surface is then gently washed to remove only the non-annealed DNA, and a ligase is added to connect the DNA locus to the oligonucleotide hairpin. At this point the DNA is covalently linked to the oligo, which itself is covalently linked to the surface, and the surface can be aggressively cleaned and heated without

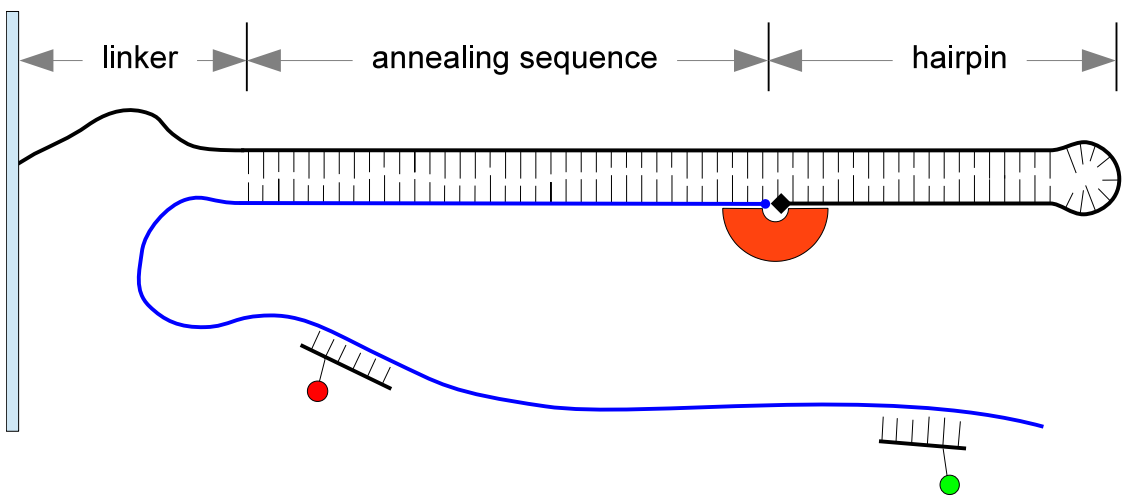

Figure 1. Covalent link through a hairpin. The hairpin construct (black) is covalently linked to the surface at left. It is phosphorylated at the 5' end (diamond), and has an annealing region which is complimentary to the genomic DNA fragment (genomic DNA in blue). Specific fragments are created by digestion of the genomic DNA with MseI. Once annealed, a ligase (red) links the hairpin to the genomic DNA. After ligation, the DNA can be labeled by annealing markers (oligo labeled with red and green circles) to the genomic fragment.

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losing the original DNA strand.

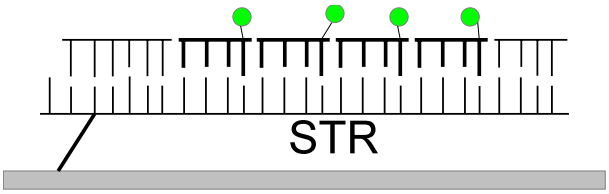

Figure 2. Strategy for determining number of STRs by labeling individual repeats. The bottom single strand is the genomic sample, while the top strand shows the labeled individual repeats and flanking primers which are annealed to the genomic DNA. As they anneal to the DNA, the primers are ligated in place for stability. The number of STRs is then determined by counting the number of labels, either by stepwise photobleaching of fluorescent dyes or by TEM imaging of gold beads.

Characterizing immobilized DNA markers

Once single stranded DNA was immobilized on the surface, we sought to determine the number of TPOX STR repeats by annealing to it oligonucleotides which are complementary to the repeats and labeled with fluorophores or beads, as shown in Figure 2. The longer the STR repeat region is, the more labeled oligonucleotides will anneal to it. The number of oligonucleotides can be determined by counting the labels directly.

Because of the short length of the repeats, the probes are not expected to remain annealed to the genomic DNA for very long. In order to facilitate measurements, eight base pairs oligonucleotides were used to increase annealing, and the probes were ligated to each other and to flanking oligos to increase stability. Reduced temperatures were also investigated to increase annealing. It is expected that abasic sites or SNP would additionally lower the annealing efficiency, but for this proof of concept work these situations were not investigated. In future work, synthetic nucleotides with higher binding affinities can be tried in an attempt to overcome issues of poor annealing.

The probe oligos were labeled either with an Alexa 488 dye or a gold bead. For this proof of concept work, we did not test abasic sites or SNPs. The number of dyes were determined via single molecule photobleaching. As long as the DNA is immobilized on the surface, it can be monitored over time. Photobleaching will destroy the fluorophores one by one, until eventually all of the fluorescent labels are destroyed.[45,46] Time traces of the data will show step-wise decrease in the signal with each step corresponding to a single fluorophore being destroyed. By counting the number steps, the number of oligonucleotides can be determined, in this case with each oligo corresponding to 2 STRs.

The same approach for labeling was used with the gold beads, but the sample was imaged using transmission electron microscopy. Here, the beads showed up clearly and the number of beads in a cluster could be counted to determine the number of oligos that annealed.

Characterizing freely diffusing DNA with Energy Transfer

As an possible alternative for quantifying the length of a locus, fluorescence energy transfer was investigated. Energy transfer exploits the fact that when a fluorophore is in the excited state, it can either emit a photon *or* transfer the energy to a nearby quencher. Transfer of energy means no photon is emitted, and the dye appears less bright. The efficiency of the energy transfer depends on the distance to the quencher, therefore brightness of the dye is a measure of the distance from the dye to the quencher. By placing the dye and quencher on opposite ends of the locus as in Figure 3, the length of the locus can be inferred from the brightness of the dye.

In practice, distance dependent quenching is complex, depending on the conformation of the molecules as well as the absolute distance. Therefore to establish a proof of concept for this

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approach, we performed these experiments in a solution which contained a large number of freely floating molecules. As such, these experiments did not immediately address the issues of low copy number of degraded DNA, although such applications can be developed with further research. However, the protocol is PCR free, involving mixing of the markers with the DNA and evaluating the results directly. The technique therefore has the potential for becoming a rapid, possibly mobile evaluation technique.

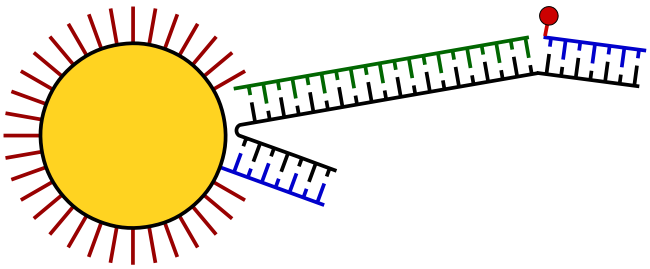

Figure 3. NSET quenching construct: two primers (blue) are annealed to a single stranded forensic DNA sample (black). One primer is labeled with a gold bead, while the other is labeled with a fluorescent dye. The brightness of the dye depends on the distance from the dye to the gold bead. Therefore, proximity dependent quenching from the dye to the bead is used to determine the length of the locus repeat region. A spacer (green) is added to keep the bead-dye distance stable. A complimentary DNA strand is shown here as the spacer, but both PEG and Ficoll were also used.

FINDINGS

Immobilization of DNA samples

Our results demonstrate that it is possible to take forensic DNA samples and chemically attach the original DNA to a surface. Doing so required fragmenting the DNA with a restriction enzyme which left specific 3' sequences. Upon deposition on a specfically modified surface, only the fragment of interest annealed efficiently and was subsequently ligated to the surface. As a test locus, we chose TPOX. To demonstrate that the TPOX gene was specifically bound, we added a solution containing two short oligonucleotides, one labeled with the green dye Alexa 488 and one labeled with the red dye Alexa 594. Both strands were complimentary to different regions of the TPOX sequence. While it possible that one or the other dye might unspecifically bind to the DNA, or to the surface, the presence of both dyes co-localized on the surface was taken as evidence of the presence of the forensic DNA. In the Figure 4 below, we show a sequence of pictures which begin with the original forensic DNA bound to the surface. At first, in image A, there is no fluorescence since the hairpin and DNA themselves contains no fluorescent labels. The next image shows the co-localization of green and red dots that indicate the presence of a TPOX strand. The fluorescent labels were then melted off the oligonucleotide and washed off the surface, and subsequently the surface was re-labeled. The lack of fluorescence after washing indicated that the markers were effectively removed from the surface, whereas the re-appearance of co-localized fluorescence after labeling the surface again indicates that the original TPOX strands were still present. Although the data clearly shows that covalently linked DNA forensic strands were stable on the surface, the current set-up did not allow a specific molecule to tracked during the washing and labeling steps. Therefore each of the areas shown is a different area on the surface. This process could be repeated many times, but the surface was degraded with each washing, and by the fourth image non-specific binding of fluorescent molecules became problematic.

Counting STRs in immobilized samples

Upon depositing sample DNA containing the TPOX locus on the surface and labeling with STR repeat markers as shown in Figure 2, images of the sample show bright dots which

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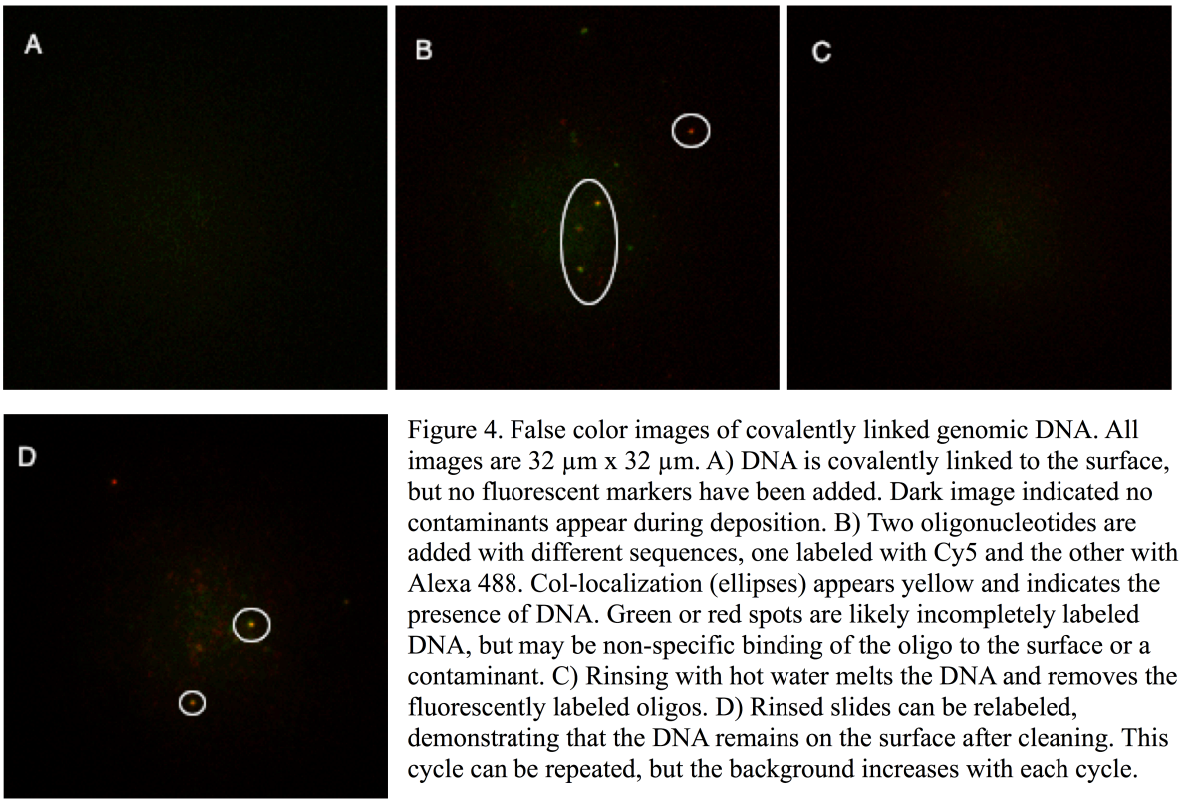

Figure 4. False color images of covalently linked genomic DNA. All images are 32 µm x 32 µm. A) DNA is covalently linked to the surface, but no fluorescent markers have been added. Dark image indicated no contaminants appear during deposition. B) Two oligonucleotides are added with different sequences, one labeled with Cy5 and the other with Alexa 488. Col-localization (ellipses) appears yellow and indicates the presence of DNA. Green or red spots are likely incompletely labeled DNA, but may be non-specific binding of the oligo to the surface or a contaminant. C) Rinsing with hot water melts the DNA and removes the fluorescently labeled oligos. D) Rinsed slides can be relabeled, demonstrating that the DNA remains on the surface after cleaning. This cycle can be repeated, but the background increases with each cycle.

correspond to the TPOX sequence immobilized on the surface (not shown). The brightnesses of the molecules was tracked over a series of 500 one-second exposures as shown on the left side of Figure 5. The vertical axis shows the brightness of the dot, whereas the horizontal axis is the time during the exposure. The total signal (white line) is the sum of several neighboring pixels (colored lines). Despite the natural fluctuation in the signal due to shot noise, a number of steps in the brightnesses are visible in each graph. Each step corresponds to a single fluorophore being destroyed, and indicates the number of repeats present. For example, in the second row, the three steps visible before background is reached indicates that there were three fluorophores present at the start of of the experiment. For reference, the brightness of neighboring pixels in the image where no DNA is present is taken as background. Since each oligo is eight base pairs long, three steps corresponds to a 6 at the TPOX site. Several different TPOX lengths were tested, demonstrating that the number of STR repeat can be determined for the TPOX gene using this photobleaching approach.

CONCLUSIONS

These results indicate that it is possible to immobilize and characterize individual DNA molecules from forensic samples using single molecule techniques. The characterization methods developed here are non-destructive to the original forensic DNA apart from the need to fragment the DNA using a restriction enzyme. Together with the covalent immobilization, these methods

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can allow the same DNA strand to be measured several times. Repeatability in single molecule techniques is essential since incomplete labeling, premature photobleaching, and artifacts in the measurement may produce incorrect results on any given analysis.

These experiments were conducted exclusively using the TPOX locus, but it can easily be extended to include several loci. There is no reason, for example, that the immobilization strategy could not simultaneously employ several different hairpin constructs, each targeting a different locus. It is worth noting that the characterization method involves annealing sequence specific oligos to the DNA evidence. Therefore, the presence of other loci with non-complimentary sequences is irrelevant. A single surface can therefore be made which covalently links a number of different loci, effectively multiplexing the approach.

The experiments here were conducted with genomic samples containing largely intact DNA. Both the immobilization technique and the characterization method can also be applied to damaged DNA, with some modification.

Implications for Criminal Justice Policy

This project consisted of basic research, exploring many alternatives for using advanced technology to determine those most suitable for forensic applications. As such the results do not have an immediate impact on policies or practices in criminal justice, except to guide future research into the most appropriate technologies. The research does demonstrate the principle that single strands of DNA can be properly genotyped under forensically relevant conditions. The

Figure 5. Counting STR repeats via fluorescence and TEM. Pictured on the left are the fluorescence signals from TPOX loci containing 6, 8, 12, and 16 repeats. The total signal is given by the white line in each graph, and is composed of the signal from several neighboring pixels (colored lines). Fluctuations are normal in any fluorescence signal, and should be filtered out to find the underlying signal (purple horizontal lines). The traces show the anticipated 2, 3, 5 and 7 steps in the signal, although as the repeat number gets higher, the steps are harder to distinguish from shot noise. In the CCD image, even a single dye is much brighter than the background. This fact allows us to use nearby pixels in the image (where there is no DNA) as a background reference. The occasional recovery of a previously bleached dye, together with decreased signal to noise in the longer loci, merited a confirmatory testing method. On the right are TEM images of the same DNA samples, but labeled with gold beads instead. The number of beads in the cluster correspond to the number steps observed in the fluorescence signal.

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natural evolution for this technology will be to develop multiplex surfaces capable of genotyping a single cell. Even before the technology is as robust as PCR typing, it will likely contribute to resolving mixtures by providing a profile for each cell individually. In these cases, there may very well be plenty of evidence, meaning that many individual cells can be tested, lowering the necessity that every analysis be error free.

Upon further refinement of the techniques, it will be possible to genotype “touch samples” present in burglaries and security breaches. The techniques here would also make it possible to genotype DNA which has been exposed to chemicals, radiation, or harsh environments.

Early applications of these methods will require specialized equipment and training. It is possible that initially only high value targets would be worth the time and manpower necessary for such analysis, but it would provide a powerful tool to help insure that those investigations don't hit a dead end. Additionally, although a single case involving “touch” DNA may not merit the cost and effort of these techniques, the high recidivism in burglary or security cases may mean that a single analysis yields progress in many cases.

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FINAL TECHNICAL REPORT

I. INTRODUCTION

Forensic analysis can reliably match minute amounts of DNA to possible donors by characterizing specific marker regions known as short tandem repeats (STR) or single nucleotide polymorphisms (SNP). Even sub-nanogram quantities of DNA can be genotyped thanks to polymerase chain reaction (PCR) technology, where a small number of DNA molecules can be repeatedly copied in order to amplify the sample up to the quantities necessary for analysis. However, even though PCR based forensic analysis has proved to be enormously successful, there are motivating factors to develop alternative methods of human identification.

For evidence samples containing just several to tens of picograms of DNA from a single individual (which also includes the case of a minor contributor in a sample which contains a DNA mixture from several donors), the stochastic variation in the pipetting and amplification of the sample can result in fluctuations of the peak heights that are actually larger than the size of a typical peak. In other words, one might expect a signal of 200 RFU's based on DNA concentration, but the actual signal could range from zero to 700 RFU's, ie. a fluctuation of 350% compared to the average peak height. Compare that percentage to a traditional non-LCN sample, where the difference in height between the two peaks at a single locus is often within 20% of their average height. For example, a 20 pg sample averages 3 double strands of DNA for each locus, but random sampling will likely result in some loci having 7 or 8 copies while others are not represented at all. Combined with the unequal amplification of each locus, and the results can show the large peak to peak height variations described above, which can make correct interpretation challenging. As a further complication, degradation of DNA samples by age, contamination with foreign substances, or exposure to the elements can hinder or prevent PCR completely.

Another point to consider is the time involved in PCR amplification. It is worth investigating analytical approaches that do not require thermal cycling, as these may reduce the time (and cost) in identifying a donor. Taking these issues together, it is evident that novel genotyping approaches which circumvent PCR would add valuable supplemental information to investigations. Failure to pursue these technologies means letting some investigative options lie dormant, possibly allowing harmful activities to continue while investigations seek to make progress.

For more than two decades now, single molecule research technology has been dramatically enhancing biological and biophysical investigations. In such research, the signal from probing individual molecules is recorded and analyzed to classify each molecule separately according to some criterion, such as structure, composition, or binding characteristics. If applied to forensic DNA evidence, this technology promises to be able to classify the genotype of a DNA sample strand by strand, and without PCR. With this capability, it is possible to imagine that genotyping low copy number samples becomes routine, or that resolving mixtures can be accomplished by typing the DNA cell by cell. Fragments of DNA may be analyzed directly, circumventing the problems of enzymatic amplification of degraded DNA samples. Finally, the potential exists to develop techniques that do not require the time or cost of PCR, providing a rapid flow through system for human identification. However, single molecule investigations are currently a basic science tool used in pristine conditions. Before such ambitious forensic goals

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can be realized, it is necessary to first identify which single molecule technologies are best suited for forensic investigations, and determine how real world forensic samples can be prepared for reliable single molecule genotyping.

In this report, we explore different strategies for typing DNA without enzymatic amplification, including fluorescence spectroscopy, atomic force microscopy, and electron microscopy. We determine the suitability of these techniques for examining single molecules of DNA, and develop protocols for preparing genomic DNA for single molecule analysis. Our results indicate that fluorescence techniques have the highest potential, and we demonstrate that it is possible to deposit genomic DNA in area sufficiently small to scan using single molecule imaging, immobilize the DNA to prevent its loss, and characterize the DNA repeatedly using single step photobleaching. Furthermore, the technique is non-destructive and allows the DNA to be retained for later confirmatory testing, enzymatic amplification, or stored for analysis with future advanced techniques. The potential for another approach, which uses energy transfer between dyes and quenchers, is also demonstrated.

Statement of the problem

DNA forensic techniques have matured to the point that minute (~ 100 pg) quantities of DNA are sufficient to reduce the number of possible contributors down to a few or a single individual.[1–3] Genotyping such minute quantities of DNA is possible due to the use of polymerase chain reaction (PCR) amplification. PCR is a cyclic polymerization process where DNA double strands are melted into two single strands, each of which is re-polymerized enzymatically into a double strand. Oligonucleotide primers introduced to the sample anneal to the melted single strands, indicating sequence specific start positions for the PCR enzyme. Any stretch of DNA flanked by two primer locations on opposite strands (the amplicon) will be amplified exponentially with repeated melting/polymerization cycles. Non-specific and spurious amplifications do occur, but with much less efficiency, generating a background noise level. For genotyping, the sizes of the amplicons are determined via capillary electrophoresis. Analyzing electropherograms requires that a detection threshold is set, and only peaks that exceed this threshold are considered for the purpose of determining a profile.[3,5] Under normal conditions, PCR amplification ensures that only the marked regions are present in sufficient quantities to exceed detection thresholds.

Difficulties rarely occur as long as the amount of DNA evidence at start is above an analytical threshold (> ~ 100 pg). Yet there remain cases in which only a trace amount of DNA is available[22] or in which the DNA degradation is too severe to allow effective amplification. DNA samples with insufficient quantity for reliable analysis (as established by validation procedures) are termed LCN. Due to stochastic variation described above, at least some of the peaks in an LCN sample will likely fail to meet signal thresholds even after amplification.[10] In these cases, PCR can produce artifacts such as allele drop-in, drop-out, or significantly imbalanced signal strength between markers. Drop-in is the appearance of a new marker and can be caused by the PCR enzyme shifting its position by one set of repeats thereby falsifying the output (stutter), or by contamination with another donor.[10,11] Some practitioners classify stutter and drop-in due to contamination as two separate phenomena. This distinction may be useful when developing procedures to minimize their occurrences, but from an analysis perspective it is difficult to classify a peak as originating from one source or the other, and in this report the

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single term drop-in will be used to refer any additional peak that does not have as its source the DNA being genotyped. Drop-outs are the non-appearance of alleles which should be there but are not amplified efficiently due to the preferential amplification of one location over another.[6–9] Imbalances in the output occur due to the stochastic nature of polymerization and the fact that early fluctuations are subsequently amplified.[10] Collectively these issues mean that when the number of DNA molecules is small (low copy number, or LCN DNA), results can be produced which may be called into question.[6–9]

A separate but related problem in DNA analysis is samples which are degraded or contaminated to the point that the efficiency of PCR amplification is diminished.[12] DNA degraded by age, radiation, or chemicals often contains nicks and breaks which upon melting in the PCR cycle cause the DNA to fragment. PCR can only amplify whole STRs; it cannot piece fragments together.

Current Approaches to Problems

The best solution for too little DNA is to improved sample collection methods to ensure that enough DNA is recovered to meet the minimum quantity threshold for analyzable samples.[7,12] There are different practices for collecting DNA, varying in swab material,[13,14] wet or dry, number of swipes,[15,16] and which solvents to use.[13] Unfortunately there is little research dedicated to determining the optimal parameters for DNA collection.[39]

It is also true that there are occasions when DNA evidence simply isn't there to be conserved.[22,23] One approach when copy numbers are low is to increase the number of PCR cycles and amplify the DNA more.[6] This approach may provide results which exceed the established signal threshold, but that outcome alone does not make the analysis more reliable. The existing thresholds were established to separate signal from artifacts and validated under different experimental conditions.[5] Applying existing thresholds to new conditions is unreliable. Furthermore, this approach does nothing to counter the stochastic variations in the first few cycles which additional PCR cycles will only amplify. It is possible to try to confirm LCN results by splitting the sample and analyzing it twice or more.[6] It may be unlikely that the same stochastic variation occurs twice, but splitting the sample increases the likelihood of stochastic effects and in the case of disparate results it may not be possible to identify the “correct” result. Furthermore, by its nature LCN data does not lend itself to being split more than 2 or 3 times. Other approaches include a post-PCR purification/concentration step, improved fluorescent tags, whole-genome amplification,[18,19] or using additives which make the PCR more efficient.[20,21] Despite these advances, obtaining reliable results from LCN samples remains challenging.

DNA which has suffered damage also requires careful analysis. Realizing that the longer an STR region is then the more likely damage occurs within the STR, one solution has been to more restrictively target the STR. There has been success in moving the start and end of the PCR region closer to the repeat region, creating so-called mini-STRs which increases the chances for successful PCR.[17] However the desire to shrink the amplicon as much as possible also means that fewer loci can be investigated. Amplicons of different loci need to be of different sizes in order to prevent their lengths from overlapping in an electropherogram. Therefore only a few of the many available loci can be analyzed simultaneously using mini-STRs. Another approach is to repair DNA damage enzymatically by using ligases. Alternatively one can turn to single nucleotide polymorphisms (SNP).[40] This technology looks for mutations in a single base pair, therefore the amplified DNA region can be very small. Correspondingly, the information content

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is lower and more SNPs must be investigated in order to get a similar level of specificity in identifying individuals.[41]

Rationale for the research

Given the current difficulties in analyzing low copy number and degraded DNA samples, and the fact that such samples occur in high value crime scenes such as missing persons, national security incidents, and human remains identification, there is a critical need for new technologies which can effectively and reliably characterize LCN, degraded, and contaminated DNA evidence. This technology should fundamentally address the limitations of PCR amplification and if possible be non-destructive in order to allow repeated testing with alternative technologies.

Single molecule techniques have been developed for biological and biophysical investigations over the last 15 – 20 years, with the techniques becoming more quantitative, robust, and diverse over the last 10 years. The challenge in developing single molecule techniques was to design equipment and experiments which had the signal-to-noise ratio necessary to reliably record the inherently weak signal from a single molecule. Achieving this is now routine (for example, Figure 4). Under laboratory conditions, these techniques combine high sensitivity, specificity, resolution, and discrimination. Applying these technologies to forensic analysis of DNA promises an ability to examine DNA evidence at the molecular level without PCR amplification. Genotyping of low copy number, damaged or contaminated DNA would therefore be possible.

However forensic evidence does not come in pristine laboratory packages, and single molecule techniques, due to their exquisite sensitivity, are susceptible to contaminants. This research therefore surveys a number of approaches for preparing forensic samples and investigating them using single molecule techniques in order to determine which strategies and technologies are the most promising for future development. In particular we examine methods for immobilizing forensic DNA samples in order to allow repeated measurement of the same DNA (for confirmation of results), and non-destructive methods for determining the size of forensic locus.

II. METHODS

Several different tasks and strategies were explored in this research, with the methods for for each being organized in this section as follows:

A. DNA collection protocol for all experiments
B. Specific covalent attachment of unmodified genomic DNA
C. Characterization of TPOX locus via photobleaching, TEM, and AFM
D. Characterization via FRET, NSET
E. Single sided PCR amplification

After all methods have been described, the results for each technique are described in the third section.

1. DNA Collection and Isolation

The DNA samples required to test the strategies in this report were collected from

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participants in the research. Buccal swabs were collected, with informed consent, from volunteers within the Department of Physics & Astronomy here at the University of Kansas (KU). All samples were collected according to the guidelines set forth by KU's Institutional Review Board (IRB). Genomic DNA was isolated using Qiagen's DNA Blood Mini Kit (Qiagen, Valencia, CA) following the manufacturer's recommended protocol. Following extraction, the concentration of each was determined via a NanoDrop 1000 spectrophotometer (Thermoscientific, Wilmington, DE), which quantifies the amount of nucleic acid in solution based on UV absorbance.

2. Covalent attachment and subsequent recovery of unmodified, sequence-specific genomic DNA

A major issue with adapting single molecule techniques to investigate forensic samples is the requirement of immobilizing the DNA in the field of view of the equipment. Typically immobilization is accomplished through the use of some chemically active functional group on the molecule binding to a specially treated surface. However forensic DNA samples do not come with built in functional groups, so any immobilization strategy must be applicable to unmodified DNA.

Also at issue is the shear length of genomic DNA. Most single molecule techniques have fields of view only tens of micrometers wide at best. Physically attaching a random point on the DNA to the surface may leave the forensic loci outside the field of view. It is therefore necessary to specifically target a sequence near the forensic loci for immobilization in to keep the locus in the field of view of the equipment.

The most successful approach developed in this project uses restriction enzyme to fragment the DNA at specific locations, then targets only that fragment near the forensic location for ligation to an oligonucleotide which is pre-attached to the surface. As shown in Figure 1, the oligonucleotide is a specially created hairpin designed to have a double stranded stem which terminates at the 5' end with a phosphate group. The 3' end of the stem is longer than the 5' end, leaving a single stranded region whose sequence is complimentary to the targeted fragmented DNA. After the single stranded complimentary region, the 3' end is attached via a linker to the surface. Annealing the fragment to the single stranded region positions it for ligation to the stem, which creates a covalent link through the hairpin loop to the surface.

Custom DNA oligonucleotides:

For versatility in the experiments, the hairpin loop was constructed from three smaller sequences: two sequences which will eventually form the hairpin structure, and a third complimentary sequence which facilitates annealing and ligating the first two to each other. In this case, the hairpin structure (Figure 1) consisting of a stem-loop-stem and a single stranded genomic annealing region was constructed using the following oligonucleotides:

Hairpin DNA: 5'-TCCGCCGCGCGGTCACCTGCGCGGCGGAAAGAGAGATTCATC-3,' and
Amino-linker: 5'-CATTCAGTGAGGGTTCCCTAAG/3AmMC6T/-3'

Both sequences were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). An additional sequence, 5'-CTTAGGGAACCCTGACTGAATGGATGAATCTCTCTT-3', which

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served as the DNA template for the ligation of the two aforementioned oligonucloetides, was also purchased from IDT.

In order to have the ability to confirm sequence-specific binding of the fragmented genomic DNA to the covalently linked hairpin, the same company was used to generate labeled primers that are complementary in sequence to the genomic strand:

Cy5 primer: 5'-/5Cy5/CAGTGAGGGTTCCCTAAG -3,' and
Alexa 488 primer: 5'-GTCCTTGTCAGCGTTTATTTGCCCAAA/3AlexF488N/-3'

Upon receipt, each oligonucleotide was re-suspended, either in molecular grade (mg) water (Corning, Corning, NY) or mg water and a 1X Tris-EDTA buffer solution, pH 8 (Sigma Aldrich, St. Louis, MO), to create 100μM stock solutions.

DNA Phosphorylation:

End-labeling of the 5' ends of the hairpin DNA and the amino-linker were performed using T4 Polynucleotide Kinase (Catalog #: M0201S, New England Biolabs, Ipswich, MA) according to the manufacturers' specifications. To yield the highest final concentration of phosphorylated DNA (300 pmole/2μM), both reactions were performed using the 100μM stock solutions of the above mentioned oligonucleotides.

Coverslip preparation and hairpin attachment:

Amine-modified slides (NEXTERION® Slide H) were purchased from Schott (Applied Microarrays, Inc., Tempe, AZ). Prior to covalent attachment of the DNA with the 3'-amino group, the cover slides were first equilibrated to room temperature for ~20 minutes, cut into two equal halves, and then the backs cleaned with a foam swab moistened with mg water before placing in a small petri dish. Next, 20μL of the phosphorylated amino-linker (2μM) was added to the cover glass along with an equal volume of a phosphate buffered saline (PBS) solution (pH=8) containing 10% glycerol. To ensure that the amino-modified oligonucleotide spread over the entire surface, another coverslip that was cleaned via Aqua Regia was added on top of the DNA-PBS-glycerol solution before incubating in a humidified chamber for 20 minutes at room temperature. Following this incubation step, the top coverslip was removed and the bottom functionalized coverslip was washed three times with 500μL of a 1X wash buffer solution (1X PBS and 0.05% Tween 20). The ligation of the phosphorylated hairpin DNA to the covalently attached amino-linker was then performed on the slide using T4 DNA ligase (Catalog number: 15224-090, Invitrogen, Grand Island, NY) as follows: 12.2μL of the phosphorylated hairpin DNA, 12.2μL of the DNA template for ligation, 10μL of T4 DNA ligase buffer, 2μL of T4 DNA ligase enzyme and 24.4μL of mg water. Again to ensure complete coverage of the slide, an Aqua Regia cleaned coverslip was added on top. This reaction was allowed to proceed overnight in the fridge at 4°C.

Fragmentation of genomic DNA:

Human genomic DNA (~500ng) was fragmented into sequence-specific pieces using either MseI (Catalog #: R0525S, New England Biolabs, Ipswich, MA) or PacI (Catalog #: R0547S, New England Biolabs, Ipswich, MA) according to the manufacturers' recommended

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protocol. To ensure complete digestion of the genomic DNA, 40 Units of MseI was used per 50μL reaction. The restriction digest was incubated at 37°C for 1 hour followed by heat inactivation of the enzyme at 65°C for 20 minutes. Following digestion, 20μL was run on a 2% agarose gel in 1X TAE running buffer along with 10μL of undigested genomic DNA to confirm that cleavage took place and that it was complete.

Immobilization of genomic DNA:

Upon completion of the hairpin-amino-linker ligation, the coverslip was placed in ~20mL beaker of hot water (ensuring the slide is completely immersed) for 15 minutes to remove the DNA template strand that was required for proper ligation. After heating, the cover glass was returned to its petri dish. Prior to ligation of the MseI digested genomic DNA, the digested product was heated to 95°C for 5 minutes to denature the double strands. A total of 20μL of the denatured DNA was then added to the cover slide along with 10μL of T4 DNA ligase buffer, 2μL of T4 DNA ligase enzyme and 18μL of mg water. As before, an Aqua Regia cleaned coverslip was added on top to ensure complete coverage of the slide during the 4 hour ligation in a humidified chamber at room temperature.

Tagging of immobilized DNA:

Following ligation of the genomic DNA, the top cover slip was removed and the functionalized cover slide with the hairpin+genomic DNA washed two times with 500μL of 1X wash buffer and once with 500μL of mg water. The cover slide was then placed in ~20mL of mg water for 5 minutes to ensure than any unbound genomic DNA was removed. Next, the complementary Cy5 and AF488 labeled primers (1μM concentration of each) were added to the cover slide, followed by an Aqua Regia cleaned cover slip on top. The primers were allowed to anneal to the genomic DNA attached to the hairpin for 1 hour in a humidified chamber at room temperature. After incubation, the top coverslip was again removed and the cover glass with the hairpin and genomic DNA was wash as above before blow drying with nitrogen. The prepared coverslip was stored at 4°C in a petri dish that was sealed with parafilm until imaging.

Imaging via Total Internal Reflection Fluorescence (TIRF) Microscopy:

The Cy5 and AF488 dye molecules attached to the complementary primers were excited using BHL-600 (Becker & Hickl GmbH, Berlin, Germany) and P-C-485 pulsed diode lasers (PicoQuant, Germany), respectively. Each laser beam was coupled into a 2 m long polarization maintaining single mode fiber (PM-S405-XP, ThorLabs, Trenton, NJ) before being focused onto the back focal plane of an Apo 1.22 NA 60X water immersion lens (Nikon, Melville, NY). TIRF was then achieved by translating the beam sideways until the exit angle from the objective exceeded the critical angle for the coverslip-medium interface. The same objective was also used to collect the fluorescence emitted from the sample. The fluorescence signal was separated from the internally reflected laser using and FF502_670-Di01 dichroic mirror (Semrock, Rochester, NY). For Cy5, the fluorescence signal was bandpass filtered using a FF01-692/40 (Semrock, Rochester, NY, USA), while an FF01-525/40 filter was used (Semrock, Rochester, NY, USA) for the AF488. The filtered signals were then detected by an Andor iXon3 EMCCD camera (Andor Technologies, UK) whose spatial resolution is 160nm per pixel.

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Washing tagged DNA:

To ensure that the genomic DNA is properly ligated to the covalently attached hairpin-amino-linker structure, we removed the complementary labeled primers by placing the coverslip in ~20 mL of hot water for 15 minutes. The cover slide was then dried with nitrogen and imaged via TIRF to ensure that the fluorescent molecules previously observed are no longer present. Once this was confirmed, the Cy5 and AF488 primers were added to the cover glass as previously mentioned and incubated for one hour in a humidified chamber before washing, drying and re-imaging.

Release of immobilized DNA from surface:

The amino-linker hairpin structure was designed with a HinfI restriction site within the genomic annealing region so that the genomic DNA, once measured and typed repeatedly, can be released from the coverslip for traditional analysis, if so desired. It should be noted that prior to the restriction digestion, the cover slide was cut into two halves, one for the HinfI digestion and the other for direct PCR analysis (broken into smaller pieces first). To release the genomic DNA from the covalently attached hairpin, restriction digestion with HinfI was performed directly on the slide as follows: 3µL HinfI enzyme (Catalog #: R0155S, New England Biolabs, Ipswich, MA), 5µL CutSmart Buffer and 42µL of mg water. The reaction was allowed to proceed at 37°C for 60 minutes before heat inactivating the enzyme at 80°C for 20 minutes. Following digestion, 25µL of water is added to the coverslip, mixed up and down with the pipette and then added to a 0.2mL tube for downstream PCR analysis.

PCR amplification of genomic DNA:

PCR amplification of the HinfI-released DNA as well as of the coverslip with the covalently attached hairpin-amino-linker was performed using in a Techne 3PrimeG thermal cycler (Bibby Scientific Limited, Staffordshire, UK) using the TopTaqTM Master Mix kit (Qiagen, Valencia, CA) and the previously published primer sequences for the TPOX locus (Huang, Schumm and Budowle, 1995). Cycling conditions were as follows: (1) 95°C for 5 minutes, (2) 94°C for 30 seconds, 54°C for 30 seconds; 72°C for 1 minute for a total of 35 cycles, and (3) 72°C for 10 minutes. The amplified products were then run on a 2.5% agarose gel (1X TAE running buffer) and compared with PCR of the MseI digested DNA as well as regular genomic DNA.

3. Counting the number of STR repeats via Photobleaching, TEM and AFM

In attempting to identify an individual from a single cell's DNA, it is necessary to have techniques that are either very reliable or very repeatable, preferably both. The most successful strategy developed here was to tag the tandem repeats within a locus with a marker that was clearly visible above the background. Counting the number of markers then revealed the genotype at that locus.

Both fluorescent labels and gold beads were used as markers, the former for single molecule photobleaching and the latter for transmission electron microscopy and atomic force microscopy. For these experiments, it was necessary to create small oligonucleotides which were complimentary to the STR repeat sequence and to label them with the desired marker. Ideally,

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four base pair oligos would be created and labeled, but such short oligos would create annealing stability issues. Therefore eight base pair repeats were created and labeled. While the fluorescence labeling is fairly straightforward, labeling with the beads required the beads to be attached via a linker to put the beads far enough from the oligo to prevent steric interactions with neighboring beads.

Custom DNA oligonucleotides:

For this set of experiments, we examined the TPOX locus, one of the fifteen autosomal STR loci routinely typed in forensic casework. The mini-STR primers published in Butler et al. (2003) were used to gather the reference sequence (Accession # M68651) from GenBank® (http://www.ncbi.nlm.nih.gov). Template sequences (5'-CTTAGGGAACCCTCACTG[AATG]n TTTGGGCAAATAAACGCTGACAAGGAC-3') containing 4, 6, 8, 12 and 16 AATG repeats, a range that encompasses the entire repertoire of repeats at this locus, were synthesized by Integrated DNA Technologies (IDT Inc., Coralville, IA). The same company was also used to generate the two complementary flanking primer sequences, both of which included one tetranucleotide repeat (5'-/5'Phos/CATTCAGTGAGGGTTCCCTAA G-3' and 5'-GTCCTTGTCAGCGTTTATTTGCCCAAACATT-3'), a phosphorylated 8bp repeat sequence modified with an azide on the 4th base (5'-/5'Phos/CAT/iAzideN/CATT-3'), and a 38bp linker strand modified with hexynl and thiol groups at the 5' and 3' ends, respectively (5'-/5Hexynl/GTAGTGACGCTATGTGATCGAGATATCGTATTTTTTTT/3ThiolMC3-D/-3'). In addition, another phosphorylated 8bp repeat sequence tagged with an Alexa Fluor 488 dye via an amino C6 linker on the 4th base (5'-PO4-CAT [AmC6~dT+ Alexa488] CATT-3') was synthesized by Eurofins (Euorfins MWG Operon, Huntsville, AL). All oligonucleotides were re-suspended in molecular grade (mg) water (Corning, Corning, NY) water and 1X Tris-ethylenediamine tetraacetic acid (TE) buffer (Sigma Aldrich, St. Louis, MO) to create 100μM stock solutions.

Surface preparation

Standard #1 coverslips (Thermofischer, Waltham, MA) were cleaned with an aqua regia etch for 2 hours and then rinsed twice in molecular grade (mg) water (Corning, Corning, NY). After rinsing, the coverslips were placed in a coverslip rack and sonicated for 10 minutes in a beaker containing ~150mL of fresh mg water (Corning, Corning, NY). The cleaned coverslips were stored in mg water until used. Conversely, the discs of mica (SPI Supplies, West Chester, PA) were prepared immediately before coating by cleaving the top layer on both sides with Scotch tape.

To coat the coverslips and the mica, a 0.1% poly-L-lysine solution was made according to manufacturer's instructions (Sigma Aldrich, St. Louis, MO). Prior to coating, the rack containing the cleaned coverslips was removed from the beaker of water, blown dry with nitrogen gas, and finally immersed in the polylysine solution for 5 minutes. The cleaved mica, which was placed in a cover slip rack, was also submerged in a fresh solution of 0.1% poly-L-lysine for 5 minutes. Each coverslip rack was then placed in a sterile container and allowed to dry completely for two days. After drying, the coated surfaces were stored for up to two weeks in their respective containers.

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Click Chemistry:

The phosphorylated 8bp repeat modified with the azide was attached to the 38bp linker DNA with the 5' hexynl via a click reaction using the Oligo-Click-M-Reload kit (Glen Research, Sterling, VA). At the end of the reaction, 2μL of the click product was added to a 1.5mL tube containing 198μL of a 0.1M Tris-HCl solution (Fisher Scientific, Pittsburgh, PA) containing 1mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (Sigma Aldrich, St. Louis, MO) for reduction of the 3' thiol. The reduction reaction was allowed to proceed undisturbed at room temperature for 1 hour prior to the addition of a 3 fold excess (i.e., 600μL) of 5 nm gold beads (Ted Pella Inc., Redding, CA). After another hour, 1μL of a 450mM solution of O-(2-Mercaptoethyl)-O'-methyl-hexa (ethylene glycol) (Sigma Aldrich, St. Louis, MO) was added to prevent multiple DNAs from binding to the same bead.[42] The tris-TCEP-Au solution was left mixing overnight and then 40μL of a 1M NaCl solution with 100mM of Tris acetate was added and allowed to mix again overnight. The next day, the tube was centrifuged at 9,000 rpm for 30 minutes to pellet the Au-DNA monoconjugates. The supernatant, which contains the DNA not bound to a bead, was then discarded and the pellet re-suspended in 40μL of a 100mM NaCl solution containing 25mM of Tris acetate. The last three steps were repeated two more times before resuspension of the final pellet in 40μL of 0.1mM phosphate buffered saline (PBS). This protocol was adapted from Liu and Lu (2007).[43]

DNA Ligation:

Multi-labeled dsDNA constructs were generated by ligating the two complementary flanking primers and labeled 8bp oligonucleotides to the ssDNA templates with 4, 6, 8, 12 and 16 tetranucleotide repeats flanked by two primer binding regions (See Figure 1). Fifty microliter ligation reactions were performed using 1.4μL of the ssDNA template (1μM), 15μL of phosphorylated 8bp A488 tagged repeats (10μM), 10.8μL of each complementary flanking primer (10μM), 10μL of 5X reaction buffer and 2 units of T4 DNA ligase (Life Technologies, Grand Island, NY). The ligation of the reduced click product with the 5nm gold bead used the same volumes of each of the above mentioned components but the concentrations were lower (10nM of template, 100nM of the flanking primers and 100nM of the reduced click-gold DNA). The reactions were allowed to proceed for 16 hours at 14ºC before heat inactivating the enzyme at 70ºC for 10 minutes.

Gel and Capillary Electrophoresis:

To confirm the size of each fluorescently labeled ligated product, both gel and capillary electrophoresis (CE) were performed. Ten of the 50μL reaction was electrophoresed at 100 Volts for ~2 hours on a 3% agarose gel in 1X Tris-acetate ethylenediamine tetraacetic acid (TAE) running buffer. The ligated products were electrophoresed alongside the TrackIt 25bp DNA ladder (Invitrogen, Grand Island, NY) for size comparison. The gel was then stained for an hour in a 1X Gel Green solution (Biotum, Hayward, CA) and imaged on a Typhoon TRIO Imager (GE Healthcare Life Sciences, Piscataway, NJ) using the blue (488 nm) laser and 520 nm band-pass filter. Once the sizes were confirmed, an additional 25μL of each reaction was run on a 1% agarose gel which was then illuminated with a UV box so that the bands of interest could be excised from the gel. These bands were purified using the GenElute™ Gel Extraction kit (Sigma

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Aldrich, St. Louis, MO) and the final product quantitated with a NanoDrop 1000 spectrophotometer (Thermoscientific, Wilmington, DE).

Capillary electrophoresis was performed on an ABI 3730xl genetic analyzer (Applied Biosystems, Foster City, CA). Preceding CE, up to 10μL of the purified product was diluted to a final concentration of 1ng/μL. For analysis, 2.1μL of each sample was then added to a 96 well plate along with 9.15μL of a master mix containing Hi-Di Formamide, which denatures the dsDNA, and GeneScan Liz 500 (Applied Biosystems, Foster City, CA), the internal size standard. The resulting files were genotyped via the GeneMapper® 4.0 software (Applied Biosystems, Foster City, CA) using a custom-designed panel and bin set.

Transmission Electron Microscopy (TEM):

Prior to sample preparation, the carbon-coated copper grids (Ted Pella Inc., Redding, CA) were treated using the glow discharge program in a Q150T ES turbo-pumped sputter coater (Quorum Technologies Ltd., East Sussex, UK). A total of 4μL of each ligated click-gold DNA assemblage was added to a separate grid followed by the addition of 0.7μL of a 0.5X Tris-borate ethylenediamine tetraacetic acid (TBE) buffer solution. After removing the excess moisture with a kimwipe, another 0.7μL of 0.5X TBE buffer was added and the grid was allowed to air dry (~3 min). Each grid was then imaged using a FEI Tecnai F20 XT Field Emission Transmission Electron Microscope (FEI Corp., Hillsboro, OR).

Atomic Force Microscopy (AFM):

Each ligated click-gold DNA assemblage was diluted 100 fold and 10μL was added to a polylysine coated disc of mica along with 40μL of water. The DNA solution was allowed to sit for 5 minutes before rinsing the disc 3 times with 50μL of mg water and then blow drying with nitrogen gas. All samples were imaged in air using an Agilent 5500 AFM (Agilent Technologies, Tempe, AZ). High-resolution, 1nm silicon tips with a resonant frequency of 150 kHz were used in tapping mode to collect images of bead clusters.

Total Internal Reflection Fluorescence (TIRF) Microscopy:

Objective-type total internal reflection fluorescence microscopy (TIRF) was performed by focusing a 485nm diode laser (PicoQuant, Germany) onto the back aperture of an Apo TIRF 1.49 NA 100X oil immersion lens (Nikon, Melville, NY). The beam into the microscope was translated to the side, changing the angle of the beam leaving the objective, until the beam was completely reflected back toward the objective. Fluorescence from the ligated product was collected through the same objective, passed through two filters (FF01-525/40 and FF01-525/39, Semrock, Rochester, NY, USA), and detected by an Andor iXon3 EMCCD camera (Andor Technologies, UK), whose spatial resolution is 160nm per pixel. During readout, the image was binned into a 2x2 pattern to increase the signal to noise.

Photobleaching:

For each repeat length, a total of 4μL of each purified fluorescently labeled ligated product (final concentration of 50-100pM) was deposited on a separate polylysine coated coverslip. Next, a coverslip that was cleaned as described above and dried with Nitrogen was then placed on top of the functionalized coverslip to aid in bringing the molecules as close to the

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surface as possible. A 300 μm2 area of the sample was illuminated and monitored as described above until all fluorescence had been bleached. It should be noted here that any single fluorescent dye is orders of magnitude brighter than the background, and are therefore easily detected. Furthermore, the brightness of a given dye is typically stable, and suddenly switching the dye off (as happens with photobleaching) results in a clear and sudden drop in the brightness of the pixel (see Figure 5). Prior to every measurement, the sample was rotated to a new region of the slide to prevent premature photobleaching. Each ligated product was imaged using a power of 10μW for a total of 750 consecutive frames, with each frame having a 0.25 to 2 second exposure time.

Analysis:

Software written in-house using LabVIEW 8.6 (National Instruments, TX) was used to compile time traces for each pixel from the 750 frames and identify candidate pixels from the amplitude of the autocorrelation function of the time trace. Time traces from neighboring pixels were checked for similarity to the candidate pixel: if the neighboring pixel only had features (statistically significant variations in intensity) that were correlated with features in the candidate pixel, the data from the two pixels was added together. If the neighboring pixel had any features different from the candidate pixel, both were excluded in order to avoid the possibility of crosstalk between different molecules too near to each other on the surface. Neighboring pixels with no features were not considered further. The number of steps in a time trace was determined from the number of unique plateaus levels that could be identified in the time trace. However, because the intensity traces can be noisy, particularly as the number of fluorophores on the DNA molecules increase, the Chung-Kennedy filter (Chung and Kennedy, 1991) was applied to average out the noise and reveal more discernible steps.

4. Determining STR length via NSET and FRET

In select cases, the quantity of DNA is not an issue, but rather it is too contaminated or damaged to be effectively amplified by PCR. We therefore investigated methods for quantifying dissolved, freely diffusing DNA as well. This approach is not suitable for low copy number DNA due to the low frequency with which the DNA will enter the field of view of the microscope, but it is suitable for higher concentrations of DNA. In this approach the DNA is labeled with two markers, one at each end of the tandem repeats, and the distance between the markers is determined via near surface energy transfer (NSET) and FRET. In addition to facilitating the analysis of degraded samples, a robust technique of this sort may also permit the development of fast identification technologies which do not require PCR cycling in those cases where there is ample evidence.

Custom DNA oligonucleotides:

The reference sequence for the Human thyroid peroxidase (hTPO) gene (Accession # M68651) was gathered from GenBank® (http://www.ncbi.nlm.nih.gov) using the mini-STR primers published in Butler *et al* (2003). Template sequences containing 3, 4, 5, 6, 8, 11, 12, 13, 16, 18 and 35 AATG repeats including Bulter's TPOX forward and reverse mini primers were purchased from Integrated DNA Technologies (IDT Inc., Coralville, IA). The same company

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was also used to synthesize complementary TPOX forward (5'-/5Cy5/ CAG TGA GGG TTC CCT AAG-3') and reverse (5'-GTC CTT GTC AGC GTT TAT TTG CCC AAA /3DTPA/-3') primers modified with a 5' Cy5™ fluorophore and a 3' Dithiol linker, respectively. In addition, for the FRET experiments, the later primer was reordered with an Alexa 488 fluorophore attached to the 3' end (i.e., 5'-GTC CTT GTC AGC GTT TAT TTG CCC AAA/3AlexF488N/-3'). Upon arrival, all oligonucleotide sequences were re-suspended in molecular grade water (Corning, Corning, NY) and a 1X Tris-EDTA buffer solution, pH 8 (Sigma Aldrich, St. Louis, MO), to create 100 micromolar (μM) stock solutions.

Gold Nanoparticles:

Unconjugated 5, 10, 20, 30 and 60 nanometer (nm) Au colloids were purchased from Ted Pella (Ted Pella Inc., Redding, CA). Once received, the concentration of Au nanoparticles in each solution was calculated using the number of particles present per milliliter (mL) and Avogadro's number. For the 5, 10 and 20nm beads, the concentrations were in the nanomolar range (*i.e.*, 83nM, 9.5nM, 1.16nM, respectively) while for the two larger bead sizes, the concentration was much less (330 picomolar (pM) for the 30nm beads and 43pM for the 60 nm colloids). In addition, to confirm the diameter of the Au beads in the above mentioned colloid solutions, transmission electron micrographs were taken with the FEI Tecnai F20 XT Field Emission Transmission Electron Microscope (Tecnai F20, FEI Corp) using the sample preparation protocol described in Choi, Kim, and Seo.[42]

Phosphination and preparation of Au-DNA Monoconjugates:

Bis(p-sulfonatophenyl) phenylphosphine dihydrate dipotassium (BSPP) salt (1 mg) was added to the 5 and the 10 nm Au bead solutions (3.3 mL to each) and allowed to stir gently overnight. The next day, NaCl solid was added to each BSPP-Au mixture, while stirring, until the color of the solution changed from burgundy to light purple. Each tube was then centrifuged at 3,000 rpm for 30 minutes and then at 9,000 for 10 minutes. The supernatant in each tube was then removed with a pipette and the pellet resuspended in 1mL of a 2.5 mM solution of BSPP. Methanol (1mL) was added to each tube followed by centrifugation for 30 minutes at 3,000 rpm 30 minutes and then 10 minutes at 9,000 rpm. Again the supernatant was removed and the pellet resuspended in 1mL of 2.5 mM BSPP (protocol adapted from Ding et al.).[44] Each bead solution was then nanodropped at 520 nm to determine concentration.

Prior to addition to the above mentioned bead solutions, the dithiol group on the TPOX reverse primer had to be reduced. This was performed by incubating a the TPOX reverse primer (50uM and 5uM concentrations) in a solution of 0.1M Tris containing 1mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (Sigma Aldrich, St. Louis, MO) for 60 min.

Following incubation of the reverse primer with the Tris-TCEP solution, a 5 fold excess (*i.e.*, 1000μL) of Au beads was added to each reaction. The reactions were left overnight to mix gently at room temperature. The next day, 40μL of buffer comprised of 1M NaCl and 100 mM Tris-acetate buffer was added to each reaction tube and again allowed to site overnight, stirring gently at room temperature. On the final day, the reaction tubes were centrifuged at 9,000 rpm for 30 minutes to pellet the Au-DNA monoconjugates. The supernatant, which should contain any 'free' DNA, was then discarded and the pellet re-suspended in 40μL of a solution consisting of 100 mM NaCl and 25 mM Tris-acetate. Once again the tubes were centrifuged at 9,000 rpm

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for 30 min to pellet only the DNA linked to Au. The latter two steps were repeated another two times before re-suspending the Au-DNA monoconjugates in 40 μL of 0.1 mM PBS (protocol adapted from Liu and Lu)[43].

Hybridization Reactions:

Double stranded (ds) DNA-Au-Cy5 molecules were assembled by adding 15 μL of template DNA (50 nM), 15 μL of TPOX forward primer with Cy5 (15 nM), 15 μL of reduced TPOX reverse primer with Au (50 nM) and 15 μL of NaCl (150 mM) to a 0.2 mL tube that was heated to 95°C for 2 min and then cooled in 5 degree increments (10 seconds at each temperature) to 23°C. Following hybridization, 15 μL of a 40 mg/mL Ficoll-400 solution was added to each reaction. The reactions were stored in the dark at room temperature for 26 hours prior to measuring.

For the FRET experiments, the dsDNA complexes were assembled by adding 20 μL of template DNA (50 nM), 20 μL of TPOX forward primer with 5' Cy5 (50 nM), 20 μL of TPOX reverse primer with 3' A488 (15 nM) and 20 μL of a 2 M NaCl solution (final concentration is 0.5 M) to a 0.2 mL tube that was heated to 95°C for 2 min and then cooled in 5 degree increments (10 seconds at each temperature) to 23°C.

NSET and FRET Measurements:

Fluorescent experiments were performed via confocal microscopy using a Nikon Eclipse Ti Inverted Microscope (Nikon Instruments Inc., Melville NY) equipped with a 1.2 NA water objective (Nikon Instruments Inc., Melville NY). A BHL-600 pulsed diode laser (Becker & Hickl GmbH, Berlin, Germany), which operates with a 50 MHz repetition rate and 50 picosecond pulse widths, was used as the excitation source ($\lambda = 635$ nm) for the Cy5 molecules. Conversely, the FRET experiments were performed using a P-C-485 pulsed diode (PicoQuant, Germany) laser that operates with a 40 MHz repetition rate and pulse widths of 500 picoseconds. To achieve a nearly gaussian beam profile, the laser beam was coupled into a 2 m long polarization maintaining single mode fiber (PM-S405-XP, ThorLabs, Trenton, NJ). After the fiber, the laser beam was expanded and collimated to a diameter of 5 mm prior to being focused into the dsDNA solutions on the glass coverslip above the objective.

Once excited, the fluorescence generated from the fluorescently labeled molecules was emitted back through the same objective (epifluorescence) and separated from Raman scatter using a FF502_670-Di01 dichroic beam splitter (Semrock, Rochester NY). The fluorescent signal was then allowed to pass through a FF560-Di01 dichroic mirror, followed by an FF01-692/40 emission filter (Semrock, Rochester NY) and a FF01-525/40 emission filter. After the 692/40 filter, the photons are focused onto a 200μm x 200μm SPCM-AQRH-14 avalanche photodiode (PerkinElmer, Vaudreuil, Canada) via a 50 μm pinhole (ThorLabs, Newton NJ) while the photons that pass through the 525/40 filter are focused onto two 20μm x 20μm id100-20-ULN avalanche photodiodes (id-Quantique, Carouge, Switzerland). The instrument response function (IRF) of each detector was previously characterized via direct illumination, and is regularly compared to the scatter signal from the water files generated each day of experimentation.

Time resolved data was acquired using an SPC-150 time correlated single photon counting card (Becker & Hickl, Berlin, Germany). All NSET measurements were taken for a

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total of 300 sec at a room temperature using a laser power of 20 μW while FRET measurements, which were recorded for 120 sec at room temperature, were generated using a laser power of 200 μW. Lifetime and intensity information as well as background and scatter contributions to the signal were obtained from the time decay histograms of the single photon counting data by iterative convolution of the IRF using the maximum likelihood estimator and Levenberg-Marquardt algorithms. All software utilized for this experiment was written in-house using LabVIEW 8.6 (National Instrument, Austin TX).

5. Alternative Strategies and Considerations - SLLAP

Although the project primarily was devoted to exploring single molecule technologies, looking into alternative biochemical techniques was included as a possibility. One explanation for the artifacts see in the electrophoresis plots of LCN DNA is that early errors in copying are amplified in subsequent rounds of PCR. We developed a strategy which uses only a single PCR primer initially, so that repeated cycling only copies one strand of the DNA. With only one primer, there is no exponential increase in the DNA and the quantity remains small. However all of the copies that are formed are copies of the original genomic DNA, and initial errors are not further compounded. Once sufficient copies of the original DNA have been obtained, a pair of primers is introduced to PCR the single sided product up to measurable quantities. Though we had some success with this approach, the performance of commercially available kits improved dramatically to the point that we stopped pursuing this approach. We present our results up that point here.

DNA Quantitation

Given the overall importance of quantitation accuracy, particularly when dealing with low level templates,[45] the NanoDrop measurement was confirmed with the aid of a Qubit® 2.0 Fluorometer and the Qubit® dsDNA High Sensitivity (HS) Assay kit (Invitrogen, Carlsbad, CA). Following quantitation, the sample was diluted from its original concentration of 3 nanograms per microliter (ng/μL) to 750 pg/μL and 28 pg/μL.

Primer Design

Previously published primers for TPOX and FGA were obtained from the Short Tandem Repeat DNA Internet Database (STRBase) available at http://www.cstl.nist.gov/strbase/. The same site also provided the GenBank Accession Numbers for each locus' gene sequence (M68651 for TPOX and M64982.1 for FGA) , which we utilized in the current report to design extended primers, *i.e.*, those outside of the primers previously reported.[46,47] The extended TPOX (5'-ATCACTAGCACCCAGAACCGT-3') and FGA (5'- AATCCAATATGTTCAAGTCCCTTG -3') primers were synthesized by Integrated DNA Technologies (IDT Inc., Coralville, IA) and, upon delivery, were re-suspended in TE and water to generate 100 micromolar (μM) stock solutions.

PCR Amplification & STR Typing

Pre-amplification PCRs were performed in a Bio-Rad Gene Cycler™ (Bio-Rad Laboratories, Hercules, CA) using the TopTaq™ Master Mix kit (Qiagen, Valencia, CA). The final reaction volume was reduced from the recommended 100 μL to 10 μL, with the volumes of

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each of the individual kit components adjusted accordingly. In addition, the Q solution, which the manufacturer recommends to use with difficult templates, was also included. Cycling conditions were as follows: (1) 95°C for 5 minutes, (2) 94°C for 30 seconds, 54°C for 30 seconds; 72°C for 1 minute and (3) 72°C for 10 minutes. To keep the overall amount of pre-amplification the same, the number of PCR cycles varied from 62 for SLLAP-PCR, which uses only one extended primer per reaction, to only 5 cycles when using conventional symmetric-based techniques. The number of SLLAP cycles used was determined by considering how much linear amplification would be needed for a single copy of the DNA to reach a concentration above the LCN threshold (*i.e.*, > 200 pg). Assuming the each diploid cell contains 6.6 pg of DNA, one side of a single strand would need to be copied 58 times. To make a direct comparison with symmetric PCR possible, the number of cycles was increased to 62 cycles (for a total of 64 template molecules). For the symmetric based PCR pre-amplification, 5 cycles also results in 64 DNA template molecules for the STR typing reaction.

The resulting pre-amplification products were subsequently amplified in an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) using the AmpF*l*STR® Identifiler Plus™ kit (Applied Biosystems, Foster City, CA) according to the manufacturer's specifications (29 cycle variation). Following PCR amplification, STR fragments were analyzed by capillary electrophoresis on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA) using GeneScan Liz 500 (Applied Biosystems, Foster City, CA) as the internal size standard. The fragment analysis files generated were genotyped with the GeneMapper® 4.0 software, which designates alleles through comparisons to the allelic ladder provided with the kit.

Statistical Analysis

In this study, any peaks falling below the analytical threshold (AT), which was set to 75 relative fluorescence units (RFUs),[48] were not considered as true alleles. The threshold for the background noise was established, on a per locus basis, by calculating the median and standard deviation of all peaks exhibiting a height greater than 5 RFUs but less than 75 RFUs in each electropherogram. The limit of detection (LOD) noise threshold was then set as three standard deviations above the median and any peaks below this threshold were deemed noise and not considered further. Any allelic positions in the electropherograms that are below the analytical threshold are considered drop-outs. Peaks with RFUs above the LOD but below the analytical threshold were not scored as valid alleles, but were included in the computations for average peak heights (APH) of the alleles.

III. RESULTS

1. Covalent attachment and subsequent recovery of unmodified, sequence-specific genomic DNA

As described above, a hairpin designed to have a single stranded region complimentary to the MseI restriction enzyme site in the TPOX gene was created and covalently attached to a glass coverslip (Figure 1). Genomic DNA digested with MseI as described was applied without purification to the hairpin modified surface. Although MseI cuts genomic DNA at over 10^6 positions, only the cut that occurs at the TPOX gene will have the proper sequence to match the design of the hairpin.

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We chose an amount of DNA for deposition which would yield DNA strands in the field of view roughly 20% of the time. Given a field of view of 25 μm^2, and a protocol that deposited DNA over the whole 22mm x 22mm coverslip, 33000 copies of the locus were needed, equivalent to 196 ng of genomic DNA. This very large amount of genomic DNA is entirely a product of the experimental set-up, which required deposition over the entire coverslip even though only a tiny portion is imaged. Microfluidics and a redesigned imaging stage will guarantee that low copy number samples can be used and still end up in the field of view.

After allowing the fragmented DNA to anneal to the hairpin surface, a ligase was added to create a covalent link between the hairpin stem and the genomic DNA. A subsequent wash of the surface removed unligated fragments from the surface. Images taken after the washing (Figure 4A) indicate no fluorescence signal, demonstrating that neither the genomic DNA sample, nor the fragmentation protocol, nor the deposition and ligation protocols contribute unwanted background to the images.

Green and red labeled oligonucleotides were added and allowed to anneal to the covalently attached TPOX fragments, followed by a rinse which removed excess labels. Images of the labeled surface showed both red and green markers remaining on the surface. Since control images using DNA which did not contain the TPOX gene did not show any fluorescence, we conclude that the presence of the fluorescence markers indicated the presence of the TPOX fragment. Furthermore, the length of the DNA fragment (~ 80 nm) is smaller than the 160 nm spatial resolution of the camera, so fluorescent primers annealed to the same DNA strand will appear in the same pixel. Therefore, co-localization of both the red and green labeled primers is interpreted as indicating the presence of DNA containing the TPOX locus. The vertical ellipse in Figure 4B displays the co-localization of both colors as a false yellow color. Occasionally, either red or green pixels were visible separately as shown in the circle in Figure 4B. The presence of a single color may be the result of incomplete labeling of the primers with the dyes, incomplete labeling of the DNA with the primers, non-specific binding of primers to the DNA, or perhaps contaminants. Of these options, incomplete labeling of the TPOX locus with probe primers is the most likely as control images with hairpins only or hairpins plus DNA lacking the TPOX gene did not show these artifacts. For presentation here, only the comparatively rare images in which more than one co-localized pair of primers were visible were selected in order to increase the confidence that co-localization was neither a random coincidence nor an artifact of aligning the separately recorded green and red images.

After imaging the red and green tagged DNA, the surfaces were cleaned as described above to remove the fluorescent primers. Figure 4C demonstrates that the images returned to background levels after cleaning, demonstrating that the oligos used to label the genomic DNA can be removed from the genomic DNA efficiently. In order to establish that the genomic DNA itself was still attached to the surface, the DNA was again labeled with green and red oligos. The reappearance of co-localized red and green markers in Figure 4D indicate that the cleaning did not remove the covalently attached DNA, despite removing virtually all of the oligonucleotide labels annealed to the DNA. Together, the images in Figure 4 verify that genomic DNA can be immobilized and repeatedly probed and washed. This sequence was repeated three to four times before the surface quality degraded to the point that DNA could no longer be distinguished from background and non-specific binding of the labeled oligonucleotides.

To verify that ligation was necessary for retention of DNA on the surface, genomic DNA

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was annealed, but not ligated, onto the covalently linked hairpin structures. These genomic DNA samples were also labeled as before with fluorescent primers and subsequently washed to remove the unattached primers. Interestingly, the imaging process revealed that genomic DNA was present on the surface, but at a substantially lower density than when the DNA was ligated to the hairpin (data not shown). The lower surface coverage is likely the result of removal of a percentage of the genomic DNA during the rinsing step that followed labeling with the primers. As with the ligated samples, washing the surface to remove the primers left a surface with no visible fluorescence. However, in contrast to the ligated samples, a second round of labeling with fluorescent primers indicated no DNA present. Apparently, removing the initial fluorescent primers via the washing step also removed the unligated genomic DNA. Therefore we conclude that the covalent link established during ligation is essential to prevent DNA from being removed from the surface.

The hairpin surface protocol was modified to enable the attachment of damaged DNA as well as low copy number DNA. The strategy of ligating DNA to a hairpin requires complete complementarity between the DNA fragment and the hairpin as shown in Figure 1. For damaged DNA, it may be that the locus is broken between the restriction site and the STR repeats. In this case, the fragment may still anneal to the hairpin, but a gap will be left between the 5' end of the hairpin and the 3' end of the broken DNA fragment as shown on the left in Figure 6. In order to ligate such fragments, the gap between the 5' phosphate on the hairpin and the 3' end of the fragment must be filled. One possibility for filling the gap is to add dNTPs and a Taq polymerase, with the intent of filling the gap as shown on the right in Figure 6. To test this approach, we created a hairpin-genomic construct with a 10 bp gap using an alternate hairpin structure and PacI digested genomic DNA. After the addition of dNTPs, Taq polymerase, and 1 complete cycle of PCR at 54°C, the ligation reaction was performed as before. Subsequent washing of the sample and labeling with the fluorescent primers produced co-localized red and green signals as before (data not shown). This result indicated successful attachment of the truncated DNA fragments. Together with the previous result that ligation is necessary for successful imaging, we conclude that that the polymerization step was successful in filling in the gap. These findings indicate that this attachment strategy can be used to immobilize and covalently link damaged DNA to the surface as well as healthy DNA.

Finally, covalently linking genomic DNA to the surface does not preclude the use of other more traditional analytical techniques. To demonstrate this fact, we attempted PCR amplification of the genomic DNA, both while it was attached to the glass surface and after having removed it from the surface by cutting the hairpin stem with HinfI. The gels comparing the results of both approaches are presented in Figure 7. PCR amplification of genomic DNA should yield two

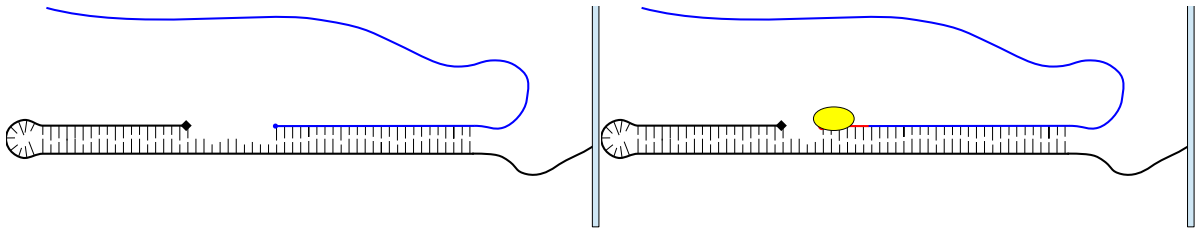

Figure 6. Covalently linking incomplete fragments. Left: Broken genomic fragments may be missing part of the annealing sequence, resulting in a gap which prevents the alignment necessary for ligation. Right: Adding a polymerase fills in the gap, leaving the pieces aligned for ligation as in Figure (1).

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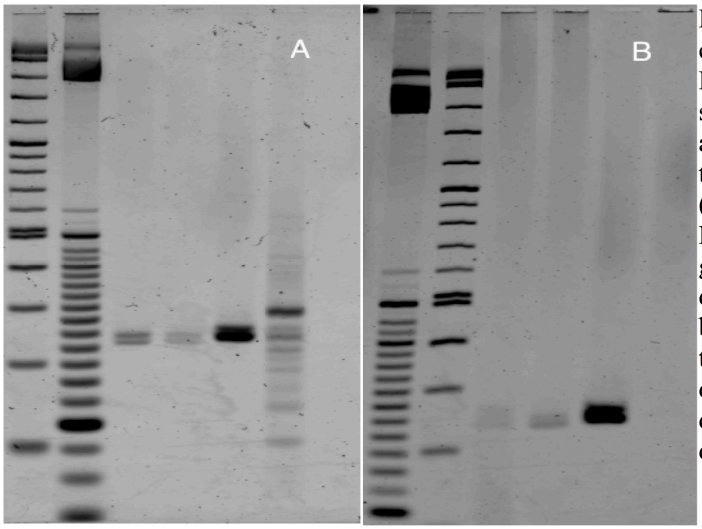

Figure 7. Recovery and amplification of covalently linked DNA. A) PCR of genomic DNA which is still covalently linked to the surface. Two bands are clearly visible at ~ 231 bp and 243 bp, as expected from sample S14. From the left: 2 log ladder, 25 bp ladder, PCR on slide (sample 1), PCR on slide (sample 2), S14 donor, MseI digest of S14, control negative. B) PCR of genomic DNA after removal from the surface by digestion with HinfI. The same two bands as before are visible at ~ 231 bp and 243 bp. From the left: 2 log ladder, 25 bp ladder, PCR of HinfI digestion of slide (sample 1), PCR of Hinf digestion of slide (sample 2), S14 donor, MseI digest of S14, control negative.

distinct bands, one at 231bp and the other at 243bp. Regardless of whether or not the DNA remained attached to the slide (Figure 7A) or was cleaved with HinfI (Figure 7B), the same two bands were observed. We did note that, after cleaning the slide, a second attempt to amplify the genomic DNA attached to the slide produced a non-specific band at ~250bp (data not shown).

2. Counting the number of STR repeats via Photobleaching and TEM

In this section, we demonstrate that ligating individual repeat units to the STR region of the locus is an efficient strategy for labeling genomic DNA, and that both the fluorescence photobleaching approach and TEM with gold beads method are effective at characterizing the number of repeats that are attached at the TPOX locus of a genomic sample. We used both synthetic and genomic DNA in this study; the synthetic DNA allowed us to test a wider variety of known genotypes, while the genomic studies demonstrated applicability to real world samples.

Double stranded DNA was constructed which had a marker on every other STR by melting double stranded DNA and then annealing appropriate oligonucleotides to the single

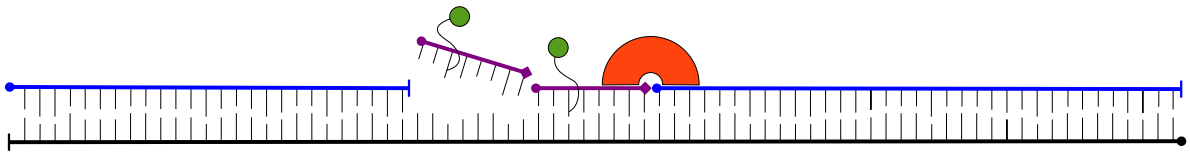

Figure 8. Oligonucleotides flanking the TPOX locus (blue) and fluorescently labeled 8 bp repeats (purple) were annealed to the target DNA strand to be probed (black). The 5' phosphate groups indicated by the diamonds allowed the repeats to be ligated in place with a DNA ligase (red). The 4 bp repeat will anneal everywhere on the DNA where there is a complimentary sequence (not just at the locus of interest), but only those probes which get ligated to the larger flanking probes will be stable enough to remain. A CCD image will have bright spots corresponding to the labeled DNA on an otherwise dark background (Figure 4 is a different experiment, but it does demonstrate that a single dye produces a bright signal well above the background). The dark pixels serve as a reference background level. Both genomic and synthetic DNA were probed. An identical strategy was used to attach gold particles to the repeats instead of the fluorescent dye, except that the gold particle was attached via a 50 bp linker to the 8 bp repeat in order to prevent steric occlusion between the beads.

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strands as shown in Figure 8. Two complementary flanking primer sequences and labeled 8bp repeat oligonucleotide were annealed to ssDNA whose sequence included a STR repeat region flanked by two primer binding regions. The repeats were labeled either with Alexa 488 or gold nanobeads, as described in the methods. Once annealed, the 8bp repeats, which contain a phosphate group at the 5' end, were ligated in the 5'-3' direction to the flanking primers.

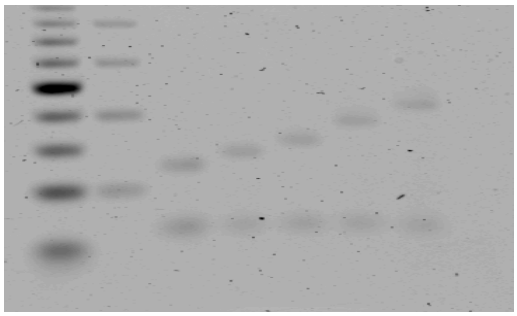

Figure 9. Agarose gel electrophoresis of the double stranded ligation product using templates with 4, 6, 8, 12 and 16 STR repeats. The expected sizes of 61, 69, 77, 93, and 109 bp, respectively, were recovered. Columns 1 and 2 contain 25 and 50 bp ladders, respectively.

Capillary and agarose gel electrophoresis determined that the ligation procedure was a good method for labeling the repeat region as it produced a high yield of properly sized amplicons and low yields of improperly formed amplicons. The results of the analysis are presented in Figures 9 and 10. Separation of the ligated products via agarose gel electrophoresis (Figure 9) yielded dsDNA fragments corresponding to the expected sizes, i.e., 61, 69, 77, 93 and 109bp, for the templates containing 4, 6, 8, 12 and 16 STR repeats. The CE (Figure 10), in contrast, produced shorter amplicons, with dominant peaks at 38.8, 49.2, 58.7, 77.4 and 95.9bp, respectively. These truncated fragments are anticipated given that the ligation reactions were performed using one unphosphorylated primer as described in the methods section. The unphosphorylated 22bp primer is removed during analysis because, unlike agarose gel electrophoresis, preparation for the CE entails denaturation of the dsDNA into single strands prior to electrophoresis. We also observed that the electrophoresis is affected by the number of fluorophores that are attached to ligated product, shifting the position of the peak by an average of 2.3 bp per attached dye towards longer lengths. However, accounting for these differences, both the CE and agarose gel results indicated that a single size was predominantly created that corresponded to the expected number of repeats for the given sample.

Although the gel and CE results indicate a single size was dominant, the CE results did indicate a small population with either with extra or missing 8bp repeats. We hypothesize that these sizes are due to the fact that the 8bp repeats can anneal anywhere on the STR repeat region, and it can therefore occur that, instead of neatly filling in the space between the flanking regions as depicted in Figure 8, the 8bp repeats anneal in a pattern that leaves gaps or overhangs, resulting in fragments which are either too short or too long. It is noticeable that the longer TPOX loci produced higher peaks for the improper sizes, likely because a longer locus provides more opportunities for non-optimum annealing. Finally, there is a peak 22 bp longer than the dominant peak in each electropherogram. Despite not being phosphorylated, it appears that the 22 bp flanking does occasionally remain attached to the rest of the ligated product.

After ligation, the fluorescently labeled DNA samples were deposited on a glass coverslip and imaged with a CCD camera using total internal reflection. On the surface, each labeled DNA molecule in the image appears as a distinct dot (see, for example, Figure 4. Although it is a different experiment it does indicate that single dye are bright enough to be clearly visible above the background). With continuous exposure to low levels of laser light, the brightness of the dot decreases as the Alexa dyes are independently destroyed, resulting in a single clear downward

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step in the intensity time trace for each dye present. Figure 5 shows the intensity traces of molecules from several representative samples. The vertical axis is the brightness of the molecule, versus the time of the observation on the horizontal axis. Due to the high resolution, a single molecule typically appears in several neighboring pixels. The individual pixels are shown as colored traces, while the sum of these pixels is shown as a white line. As is usual for such samples, the brightness fluctuates, and these fluctuations must be distinguished from the discrete steps. Using techniques described in the methods section, the data is filtered to reveal the discrete plateaus in the signal (red horizontal lines overlaying the white trace). Photobleaching data for the fluorescently labeled TPOX templates containing 4, 6, 8, 12 and 16 STR repeats are presented. Since each primer carries a repeat and each ligated oligo is 8 bp, the number of steps should be (# of repeats – 2)/2. As expected a total of 2, 3, 5 and 7 discernible steps are noted for the 6, 8 12, and 16 repeats, respectively.

We did observe that after bleaching, some fluorophores recover, leading to a step up. It is also true that as the number of repeats increases, it becomes more difficult to discern individual steps. According to several recent reports (Kurz et al., 2013, Zhang and Guo, 2014), determining the number of photobleaching steps creates interpretation challenges as the number of fluorophores attached to a particular biomolecule or biological complex increase, typically beyond 7 dye molecules. This difficulty arises because as the number of dyes increases, so does the overall fluctuation in the signal. However the size of a single step remains constant. Eventually, the fluctuations approach the size of a single step, making the step difficult to distinguish from random variations. It is also more likely that simultaneous bleaching of multiple fluorophores occurs.

To support the fluorescence measurements, we performed TEM as a complementary technique to our photobleaching studies. In this approach, gold beads are used as the label, and are seen as clusters in the TEM images. Using this approach we are able to directly determine the number of STR repeats present in a particular template by counting the number of beads in each cluster. As Figure 5 illustrates, groups of 2, 3, 5 and 7 gold beads are noted in the micrograph for the templates containing 6, 8, 12 and 16 STR repeats, supporting the results of the photobleaching experiments. It is important to note that the DNA strand comprised of 4 STR repeats was not examined using TEM since we would only observe single beads in the micrograph, the same as if examining the bead solution without attached DNA. AFM images were also attempted using the gold beads, but the samples could not be made clean enough to get acceptable images.

3. Characterization via FRET, NSET

In order to determine whether fluorescence quenching could be used to measure the length of a forensic locus, sequences of single stranded DNA corresponding to the TPOX locus were labeled just outside the STR repeat region using primers containing gold beads and fluorescent dyes as pictured in Figure 3. Initially we measured the quenching behavior of 5 different bead sizes as a function of STR length using a complimentary stretch of DNA as the spacer, exactly as pictured in Figure 3. The experiments were repeated on different days, with the results shown in Figure 11.

The first thing one notices is that for a given bead size, the intensity falls and rises repeatedly as the amplicon size is increased. Therefore the intensity from a single bead could

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correspond to one of several possible amplicon sizes. However the exact characteristics of this up-down behavior was different for different bead sizes. This fact suggests that the signal from multiple bead sizes might be combined to yield a set of intensities which corresponds to only a single amplicon size. We note that on any given day the intensity values recorded were reproducible, but from day to day, there were quantitative differences in the measurements. This fact required that calibration curves be measured at the start of each day to interpret the data properly.

The quenching of the Cy5 labeled DNA-Au conjugates changes in a bead size and STR length-dependent manner (ranging from 93 to 20%, Figures 11A and B), a finding that is consistent with previous reports using the same dye in the presence of 1.5,[49,50] 5 nm and 10 nm Au nanoparticles.[51,52] If we compare the normalized intensities of the DNA-Au conjugates

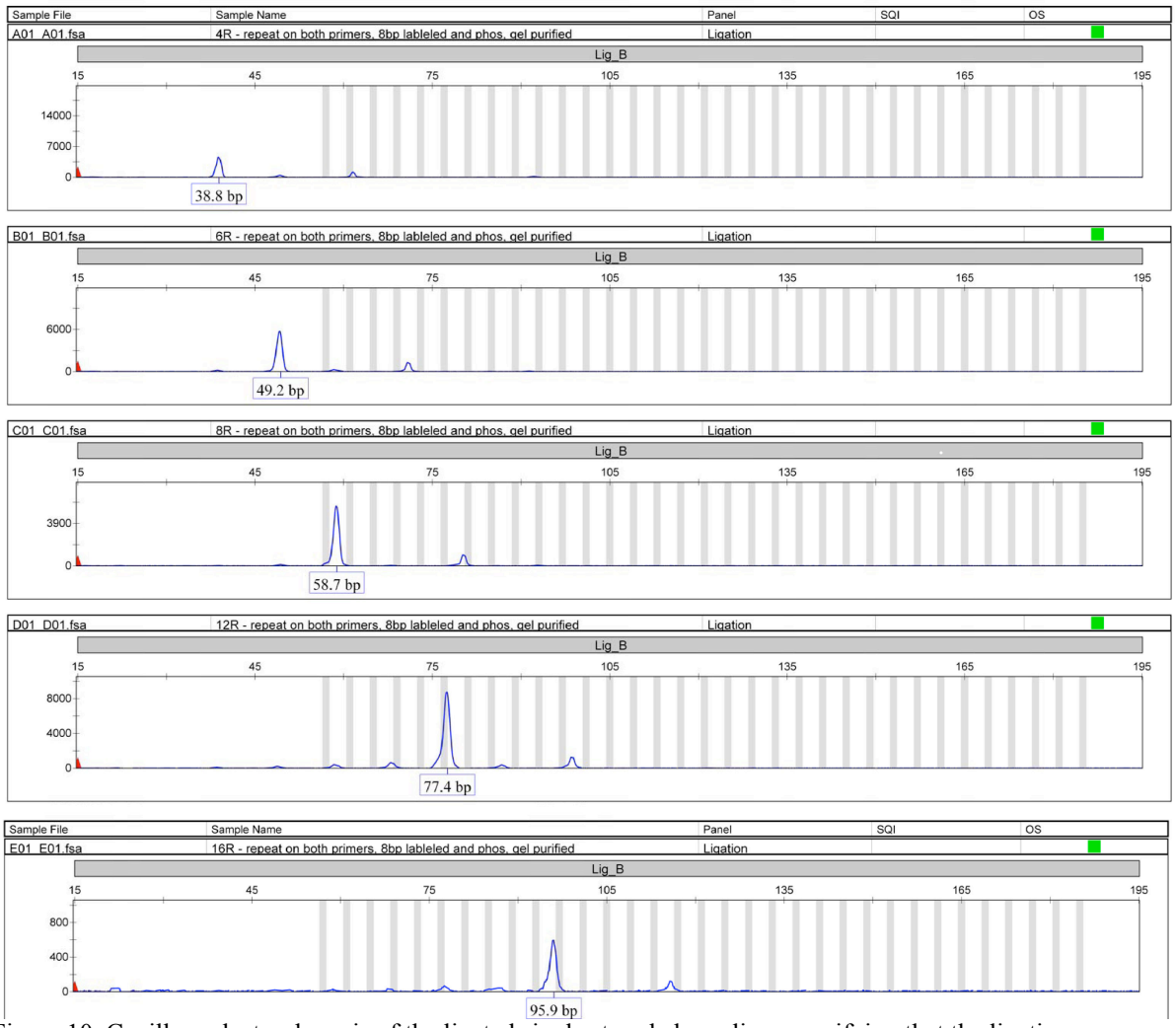

Figure 10. Capillary electrophoresis of the ligated single stranded amplicons verifying that the ligation predominantly produces a single peak with the correct number of base pairs. Lengths are shorter than the gel electrophoresis because one primer is not ligated and separates when the amplicon is melted. There is also a shifting of the peaks to longer lengths due to the extra dyes on the single strands. The grey columns represent 4 bp spacing.

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examined in this study to those published by Chhabra et al.,[51] a group that examined dsDNA templates of similar lengths labeled with a terminally located dye-Au pair, there is broad agreement among the intensity measurements, particularly among those produced using the 10nm Au nanoparticle, despite the differences in experimental setup. It is also important for us to mention that the middle spacer region, which we included to straighten out the DNA, proved to be essential as quenching showed little variation when it was absent (data not shown).

As mentioned, Figures 11A and B illustrate that fluorescence quenching of the forensically relevant DNA templates exhibits a cyclic decrease-increase behavior in the signal. The signal seems to cycle every 4 STR repeats (16 bp, 5.44 nm). We considered the possibility that this phenomenon is the result of the helical rotation of the DNA molecule itself but it is well-known that the DNA helix rotates once every 10 bp. Furthermore, if the geometry in Figure 3 is accurate, the rotation of Cy5 around a central axis should not significantly affect the overall separation between the dye and the bead, especially for the larger Au particles.

This cyclic behavior creates a challenge for forensic analysis because it is impossible to relate the measured efficiency from a single bead size to a specific STR repeat length. It must be kept in mind that at most loci there are a variety of incomplete repeat variants, more commonly referred to as microvariant alleles (e.g., a 10 at the TPOX locus will have 10 AATG repeats while a 10.2 will have the same 10 AATGs plus an additional two AA nucleotides in the sequence). According to Figure 11, several of these microvariants would have the same amount of quenching. For example, a 70% reduction in brightness by the 20 nm Au nanoparticle in Figure 11A can correspond to a DNA template containing either 8, 11.3, 12.2 or 13.3 STR repeats. However, the measurements from both days also show that the specifics of the distance dependent quenching are unique for each of the bead sizes. It is therefore possible to use more than one bead in successive measurements to clarify any ambiguity. Returning to the above example, a 30 nm bead would uniquely separate the 8 and 13.3 STR repeat samples.

We therefore attempted to use such a multi-bead approach to identify three unknown samples in a blind experiment. Three samples were chosen randomly (with replacement) from

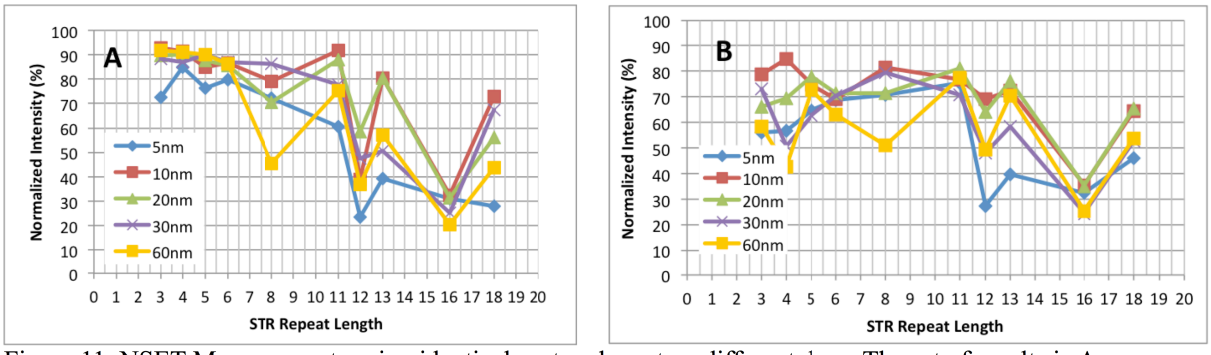

Figure 11. NSET Measurements using identical protocols on two different days. The set of results in A were taken on the first day, while the results in B were taken on a later day. Qualitatively, the signal showed a cyclic decrease-increase in the intensity, with a period of 16 bp. This fact was true regardless of the day or bead size measured. Quantitatively there is day to day variation in the exact values of the intensity, although within a single day repeated measurements were reproducible. This fact necessitated daily calibration of the signal. Because of the cyclic behavior, the use of a single bead cannot determine the STR length because several lengths can have the same brightness. However since the exact distance dependent behavior of each bead is unique, the use of multiple bead sizes can result in unambiguous combinations of values.

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nine different sizes. Due to the day to day variation, a calibration ladder was measured on each day. Due to the time necessary to set up and measure a complete set of quenching standards, only two bead sized were chosen: 5 nm and 10 nm. Figure 12 and Table 1 shows the results of the measurements from the nine standards and three unknowns, this time using Ficolli as the spacer due to the unknown lengths of the three samples. The quenching for each measurement was calculated as $Q = (1 - I / I_0)$ x 100%, where I_0 is the fluorescence intensity with no bead and I is the intensity with the bead. The dissimilarity score was calculated for each unknown relative to each standard according to :

$$D = \sqrt{(Q_{unk} - Q_{STD})^2_{5\text{nm}} + (Q_{unk} - Q_{STD})^2_{10\text{nm}}} \qquad \text{Eq. 1}$$

where Q_{unk} and Q_{STD} are the quenching values of the unknown and a known standard respectively, and the subscripts 5nm and 10nm refer to bead size in the measurement. Very dissimilar measurements yield high values for D, with the lowest D being the most likely match. The results indicate that sample F was TPOX 13, sample N was TPOX 4, and sample B was TPOX 16. These results are compiled into Table 1. Upon revealing the actual sources of the samples, it was seen that sample F was a TPOX 13, sample N was a TPOX 4, and sample B was a TPOX 11, we see that this approach was successful 2 out of 3 times. We expect that with the likelihood of a correct identification would increase with the use of more beads sizes, which would be possible in an optimized, more streamlined experiment.

We also attempted this same approach using FRET between two fluorescent dyes instead of NSET between a gold bead and a fluorescent dye. In these experiments, the green dye Alexa 488 was use in place of the gold bead, and each sample was characterized by a FRET efficiency given by

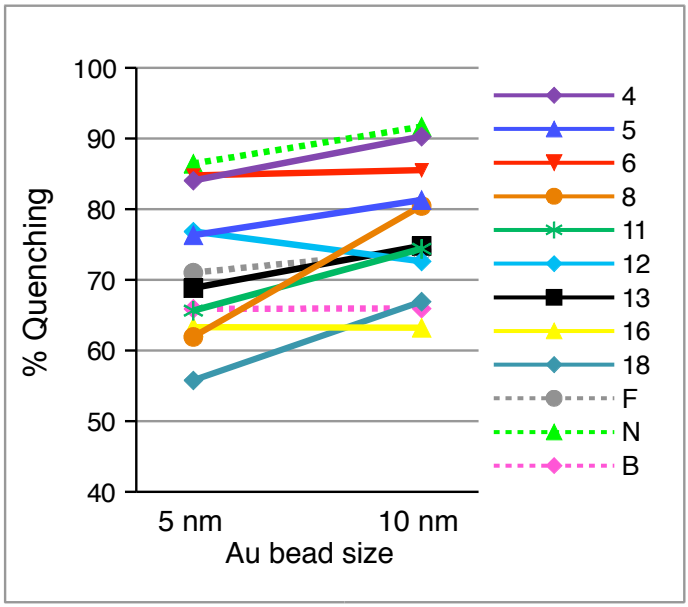

Figure 12. The results of a set of NSET quenching standards (solid line) and three unknowns (broken lines) measured for two different bead sizes, all taken on the same day.

	Dissimilarity		
Size	F	N	B
4	20.7	**2.8**	30.3
5	8.9	14.5	18.6
6	17.9	6.4	27.2
8	11.1	27.0	15.1
11	5.4	27.0	8.5
12	6.0	21.3	12.8
13	**2.3**	24.4	9.3
16	13.4	36.7	**3.8**
18	16.9	39.4	10.2

Table 1. The net dissimilarity score as computed using Eq. 1. The lowest number indicates the best match, indicated in bold. The actual values were in fact TPOX 13, 4, and 11 for samples F, N, and B, respectively.

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$$E = \frac{S_R - B_R - S_{DE} - \alpha\left(S_G - B_G\right)}{\left(S_G - B_G\right) + \left(S_R - B_R - S_{DE} - \alpha\left(S_G - B_G\right)\right)} \qquad \text{Eq 2.}$$

where the subscripts *G* and *R* correspond to the signals at the green and red detectors, and *S* is total signal and *B* is the background. In addition, S_{DE} is the signal in the red detector due to the direct excitation of the red dye by the green laser, and the crosstalk α is the fraction of photons from the green dye that end up at the red signal detector. Unlike the NSET experiments with different sized beads, the FRET measurements only have one *E* value for each standard. The unknown was called based on which standard had the closest value. The results are compiled in Table 2, along with the actual values of the unknowns. In this case, FRET was able to identify 4 of the 7 samples correctly.

Size	E	Call	Actual
4	0.661		
5	0.737		
6	0.668		
8	0.727		
11	0.708		
12	0.539		
13	0.549		
16	0.463		
18	0.335		
B	0.672	6	6
E	0.724	8	8
G	0.474	16	16
I	0.714	11	5
A	0.740	5	11
C	0.386	18	18
H	0.466	16	12

Table 2. Results for the FRET identification scheme. The FRET efficiency was calculated according to Eq 2. The top section shows the FRET efficiencies of the standards, while the bottom section shows the efficiencies of the unknowns. For each unknown, the standard with the nearest efficiency was used to determine what the sample was. The adjacent column with the actual genotypes shows that 4 of the 7 calls were correct.

4. Alternative Strategies and Considerations - SLLAP

Although our data indicated that a linear amplification treatment prior to traditional STR analysis improved the performance of the then-current STR typing technologies when working with LCN templates, the rapid improvements in commercially available kits exceeded any improvement we observed, and did so without adding additional steps to the procedure as our protocol does. By the end of the project we were no longer pursuing SLAPP, but include the results here for completeness as this work was funded by the same project.

For this work, a profile was generated using 0.75 ng of DNA, a concentration well within the limits recommended by the manufacturer. This profile revealed that the test sample possessed a heterozygous genotype at both TPOX (8, 11) and FGA (21, 24). We then analyzed LCN samples by applying three different amplification protocols to 28 pg samples of DNA from the same source. The three protocols consisted of: (1) 62 cycles of SLLAP-PCR followed by traditional STR analysis, (2) 5 cycles of symmetric PCR prior to conventional STR typing protocols for comparison, and (3) STR analysis without any pre-treatment as a control. Because of the variability known to exist with LCN samples, 30 independents reactions were performed for both the 62-cycle SLAPP-PCR and 5-cycle regular symmetric PCR samples, and 15 reactions of the no pre-amplification protocol.

A compilation of the results from 15 no pre-treatment samples and the 30 SLLAP samples is presented in Table 4 and Table 3, respectively. Table 4 shows that when analyzing the LCN samples without a pre-amplification step, TPOX yielded 2 out of the possible 30 alleles (7%) with RFUs above the analytical threshold, and FGA yielded 3 alleles (10%). In contrast,

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following our SLLAP-PCR pretreatment, the LCN samples amplified at TPOX produced a total of 43 out of a possible 60 allelic peaks that were above the AT (72%), including 17 out of 30 loci that were heterozygous (Table 3). Similar findings were also noted at the more complex FGA locus examined in this study, where SLLAP-PCR resulted in 38 alleles out of the 60 possible (63%), including 12 heterozygous loci. For both loci, the 5 cycle symmetric pre-amplification produced no peaks above the LOD.

Peak Heights

According to Figure 13 and Table 3, the ability of SLLAP-PCR to increase the peak heights at the alleles proved to be locus dependent, in agreement with previous reports.24 The peak height data summarized in Table 5 indicates that SLLAP-PCR raised the average peak height (APH) of the alleles at the TPOX locus from 63 RFUs to 683 RFUs, an 11 fold increase. All peaks above the LOD were used to calculate the APH, even those that fell below the AT. In contrast, the APH at the more complex FGA locus was increased 2.7 fold from 59 RFUs to 155 RFUs.

To verify that the increased peak heights were due to the single sided amplification specifically, and not simply to additional amplification overall, a 5 cycle symmetric pre-amplification was also attempted. This amplification step should double the original DNA 5 times, producing the same number of initial copies as the 30 cycle single sided amplification. However, when using the 5 cycle symmetric based approach, no increase in the APH was observed for either of the loci examined. In fact, no peaks at all were observed.

It should be noted that there is a notable increase in the LOD, ie. the background noise, when the SLLAP technique is applied prior to STR amplification (40% and 20% at TPOX and FGA, respectively), however this rise in the noise level is not proportional to that of peak height. These findings indicate that linear amplification of the original template DNA results in a significant increase in peak heights relative to the background noise.

Peak Height Ratio

The overall increase in APH with the SLLAP approach translates into fewer allelic drop-outs and a greater number of heterozygous genotypes at both loci examined (Table 4 through 5, Figure 13 b,c,e,f). However, despite the greater number of true heterozygotes observed, peak height imbalances remain where the height of one allele is less than 60% of the height of the dominant allele. Imbalances were noted in 11 out of the 17 heterozygous SLAPP-PCR outcomes (65%) at TPOX and in 5 of the 12 heterozygous outcomes (42%) at FGA. The peak height ratios (PHR) for each locus are noted in Table 5. For the SLAPP-PCR samples, the average PHR is calculated from all of the heterozygous loci for which both peaks were greater than the threshold. However, due to the lack of alleles in the no pre-treatment samples, PHR were calculated for loci where both peaks exceeded the LOD even if they do not exceed the AT. The average PHR at the TPOX locus after SLLAP pretreatment is noticeably lower than the other measurements. This makes sense if we consider that the lowest value a peak could have is the threshold itself, while the larger peak likely has a value near the APH. Then the peak height ratio will be small if the average peak height is many times larger than the AT, as it is for the TPOX locus after SLLAP pre-amplification.For FGA, the average peak height is only triple the analytical threshold, and the PHR is more balanced.

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Drop-out

Drop-out, where a peak does not exceed the AT, decreased as a result of the pretreatment. Prior to SLLAP pre-amplification, a drop-out rate of 93% and 90% was observed at the TPOX and FGA loci, respectively. Based on our 750 pg control samples, TPOX generates higher peaks than FGA. Previous studies suggest that higher peak heights can be expected to lead to lower drop-out rates,[53] however for our data the drop-out rates were similar for both loci. Pre-amplification with the SLLAP protocol reduced dropout to 28% at the TPOX locus and 37% at FGA. In addition, a number of alleles had visible peaks that fell below the threshold, including three at the TPOX locus and 10 at the FGA locus. This fact suggests that additional optimization of the amplification protocol would decrease the drop-out rate further, down to at least 25% at TPOX and 20% at FGA. For example, the activity of the polymerase degrades with each PCR cycle, so 62 preamplification cycles may not be the optimum number, and we expect that our higher than expected drop-out rate is caused in part by both phenomena.

Although the dominant source of both drop-out and peak height imbalance in LCN samples is expected to be the independent sampling of the alleles prior to PCR,[6,53–56] at present it is impossible for us to distinguish between drop-out due to DNA not amplified effectively versus DNA simply not being in the sample. The number of genomic copies of an allele in a sample varies according to a Poisson distribution, and can be calculated from the original amount of DNA assuming 6.6 pg per genomic copy. In our case with 28 pg of DNA (4.2 copies on average), the complete absence of an allele from the sample should only occur 1.4% of the time. However at low copy number this drop-out rate is very sensitive to the exact amount of DNA in the sample. Since precise dilutions are challenging and such low amounts are not readily quantified, it is possible that the amount of DNA is lower than our target dilution. If we assume that the 20% drop-out rate suggested above is accurate, that would correspond to a sample containing 10 pg of DNA.

Drop-in

As is common with LCN analysis, spurious alleles which are thought to result from "lab-based contamination"[57] were detected at both loci examined (Figure 13 b,e), despite the proper precautions being taken (e.g., the use DNA-free plastic-ware that was autoclaved prior to PCR, pipettes that were treated with UV before PCR setup and disposable gloves). This phenomenon, more commonly referred to as allele drop-in, was found to occur at comparable levels at both TPOX and FGA. For the TPOX locus, there were a total of 24 drop-in peaks which exceeded the AT distributed over 11 samples. Two of the drop-ins were at the -4 stutter position. FGA displayed a total of 29 drop-ins in 11 samples, including 3 drop-ins at the +4 stutter position. Many of the drop-in peaks are small compared to the dominant peak. If only peaks that are greater than 60% of the dominant peak are considered, the number of drop-ins falls to 8 for TPOX and 10 for FGA. Notably, two of the FGA samples were responsible for 16 of the drop-ins. The negative controls for all reactions performed across the FGA locus were amplicon-free, while two spurious alleles, 7.2 and 10.2, were detected in one of the control negatives for TPOX. It is worth noting that none of the above mentioned alleles can be attributed to contamination by laboratory personnel. They may, according to Steele and Balding,[58] result from "airborne DNA fragments, perhaps from previously analyzed samples."

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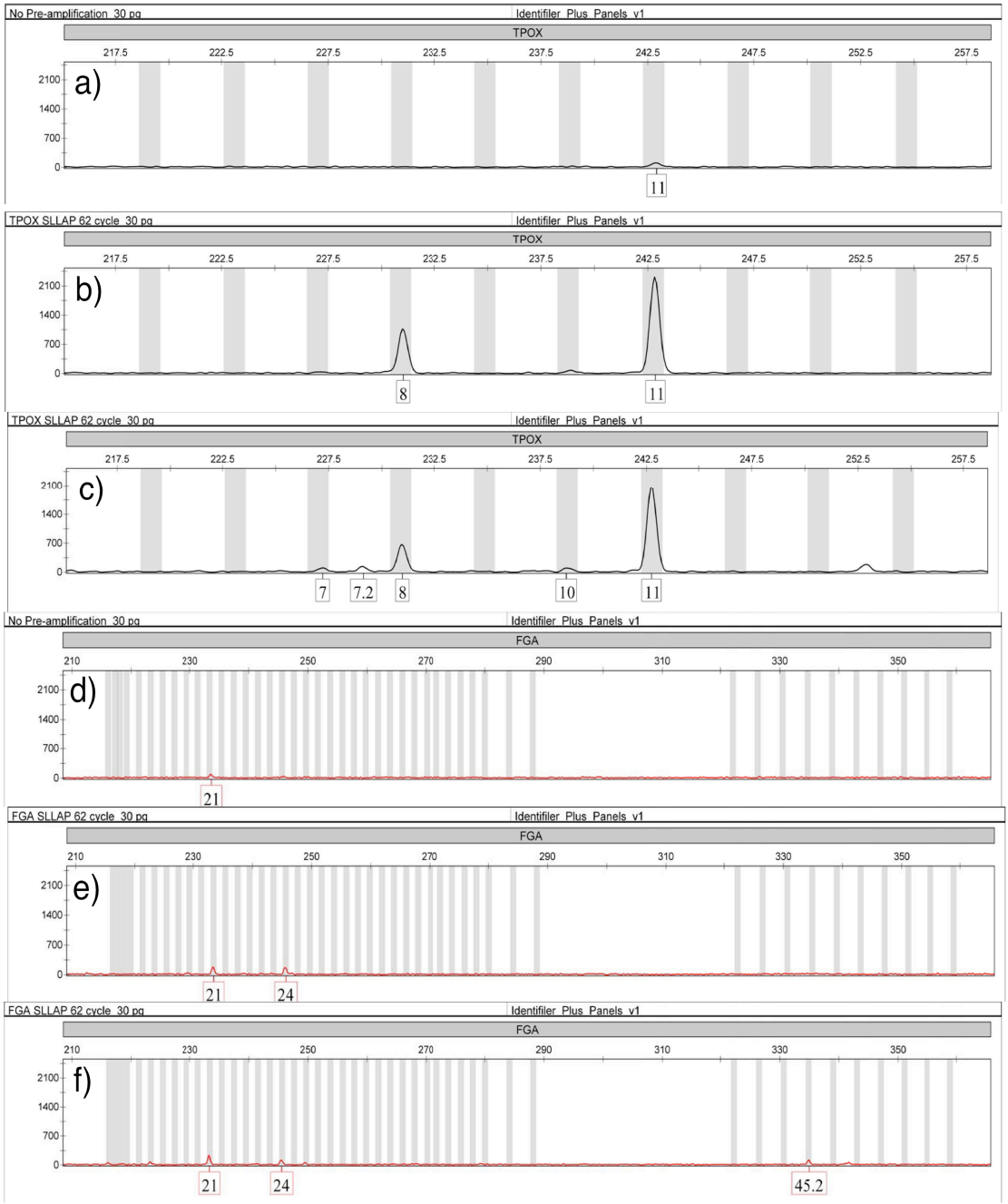

Figure 13. Profiles generated using 30 pg of DNA and either no preamplification (a & d) or the SLAPP treatment (b, c, e, f). These profiles are representative of most of the electropherograms. a) TPOX locus with no pre-amplification. Only one other profile showed a peak. b) TPOX locus showing a clean heterozygous locus. Another 13 samples were similar to this one, although the peak height ratio varied widely. Six more profiles of similar quality showed only a single allele. c) TPOX locus showing a heterozygous profile with additional drop-in. Any peaks which could be identified as pull-up from another channel were not identified as drop-in. Another 5 heterozygous profiles had drop-in, as well as two more profiles with a single allele plus drop-in. d) FGA locus with no pre-amplification. One other profile showed a heterozygous genotype. e) FGA locus showing a clean heterozygous locus. Another 7 samples were similar to this one. Eight more profiles of similar quality showed only a single allele. f) FGA locus showing a heterozygous profile with additional drop-in. Another 4 heterozygous profiles had drop-in, as well as five more profiles with a single allele plus drop-in.

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	TPOX				FGA			
Sample	Allele 8 RFU	Allele 11 RFU	PHR (%)	Drop-in alleles (RFU)	Allele 21 RFU	Allele 24 RFU	PHR (%)	Drop-in alleles (RFU)
1	1396	186	-	7 (277)	-	269	-	-
2	-	1438	-	5 (365), 10.2 (119)	190	281	67.6	45.2 (108)
3	1495	-	-	-	507	189	-	25 (2168)
4	-	133	-	5.1 (101), 8 (91), 12.1 (91)	232	131	56.5	-
5	-	-	-	-	204	-	-	17.2 (150), 37.3 (176)
6	385	184	47.8	-	-	-	-	21.1 (78), 22.1 (122), 25 (105), 35 (682), 40.2 (234), 44 (722), 48 (85), 48.1 (87), 48.2 (92)
7	1637	-	-	-	211	87	41.2	-
8	-	-	-	5.3 (1969), 9.3 (99), 11.1 (886)	-	86	-	-
9	241	-	-	-	236	118	-	45.2 (119)
10	1088	294	27.0	-	-	-	-	-
11	744	252	33.9	-	-	269	-	20.2 (15139)
12	198	-	-	-	196	-	-	13 (119), 22.1 (289), 37.1 (225)
13	420	676	62.1	-	-	119	-	-
14	672	2059	32.6	7 (104), 7.2 (143), 10 (99)	346	-	-	15 (159), 15.2 (315), 16 (85), 27.2 (196), 31.2 (158), 44.2 (577)
15	1073	2318	46.3	-	-	-	-	-
16	406	196	48.3	-	316	376	84.0	-
17	284	757	37.5	7 (232)	-	95	-	-
18	282	1047	26.9	-	92	210	43.8	-
19	198	844	23.5	-	163	91	55.8	-
20	531	1135	46.8	4.1 (109), 12.2 (314), 14 (341)	183	164	89.6	-
21	195	154	79.0	-	81	100	81.0	-
22	786	1027	76.5	-	-	-	-	-
23	461	487	94.7	-	89	77	-	25 (88)
24	-	65	-	4.1 (123), 5.2 (161)	-	214	-	42.3 (102)
25	1682	597	35.5	-	76		-	-
26	1558	563	36.1	13.3 (132), 14.2 (390)	140	101	72.1	23.1 (81)
27	-	118	-	-	108	103	95.4	-
28	1491	2929	50.9	10 (116)	-	213	-	-
29	-	636	-	-	-	172	-	-
30	650	418	64.3	-	-	170	-	-

Table 3. The results of the 30 genotyping attempts after SLLAP-PCR pre-amplification of the TPOX and FGA loci. The number of detectable peaks increases compared to no pre-amplification in Table 4. All peaks that exceeded the LOD are reported, and in the case of a heterozygous locus, the PHR is calculated. Drop-in peaks above the threshold are recorded along with their peak heights. A majority of the samples contained no drop-in, and in particular for the FGA sample the drop-ins are concentrated in a few files. Note that the TPOX and FGA data were independent reactions for a total of 60 samples; there is no connection between TPOX and FGA data.

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It is impossible for us to know if these drop-ins exist already after the SLLAP pre-amplification or first appear during the subsequent PCR analysis. Potential approaches for eliminating drop-in include post PCR purification, better SLLAP primer design, or combined SLLAP and consensus analysis. Improved primer design for SLLAP may minimize the drop-in, however if the primer was the most significant source of the drop-in we would expect to see it more often in the electropherograms. Consensus analysis after SLLAP may help identify drop-in peaks since the positions of the drop-ins varied from sample to sample. The data here indicates that the SLLAP protocol creates a sufficient number of hi-fidelity copies of the original DNA to be amplified by PCR. The consensus approach of splitting a SLLAP pre-amplified sample into 3 aliquots for PCR amplification may reveal which peaks are true alleles by appearing in at least two of the three aliquots.

IV. Conclusions

A. Discussion of findings:

1. Covalent surface attachment

This research demonstrates that genomic DNA can be specifically targeted and covalently attached to a surface, then specifically labeled for identification and quantification. The technique involves fragmenting the DNA with a restriction enzyme and depositing the cut DNA on a surface coated with a hairpin structure which will only be complimentary to the fragments near the forensic locus. Treatment with a ligase completes the covalent attachment by linking the genomic fragment to the hairpin structure. With further development, this approach shows great promise for the targeting of multiple loci in LCN and degraded DNA samples.

The work in this report used large surfaces (25 mm x 25 mm) and large amounts of DNA as a practical matter for establishing the protocol. However the instrumental techniques can only scan the surface approximately 80 μm^2 at a time, detecting only a few single DNA strands at most in any given image. This already small imaging area means there is a very high potential for scaling down the deposition to LCN quantities using microfluidics and microliter quantities of sample solutions. Furthermore, though only the TPOX locus was investigated here, there is no obvious reason why hairpins designed to target other loci cannot be designed and bound simultaneously to the surface, effectively multiplexing the attachment protocol. In those cases where single cells can be isolated, there is the potential to extract and bind the DNA of a single cell to the surface, which would allow a set of STR values from different loci to be unambiguously associated with each other. For work on LCN samples, reproducibility is essential, and this work demonstrates the ability to repeatedly label and quantify the DNA attached in this methods through several rinsing and relabeling cycles.

A further modification of the protocol demonstrated the techniques potential to target damaged and incomplete DNA fragments. The addition of a polymerase fills in any gaps between mis-cut or broken DNA fragments and the hairpin, making covalent attachment possible even for damaged DNA strands which may be missing a few base pairs. Furthermore, the annealing and ligating approach itself does not require polymerization. DNA which contains abasic sites, single nucleotide polymorphisms, and damaged nucleotides can also be covalently linked, as long as

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these issues are not so severe that the fragment can no longer anneal to the hairpin.

Finally, the protocol demonstrated that it is not necessary to choose between current PCR techniques and surface attachment methods. After covalent linking, the DNA can still be amplified via PCR, either while the DNA is still covalently linked to the surface, or a restriction enzyme has been used to cut the hairpin and release the DNA back into solution.

2. Counting STRs on a single strand of DNA

Annealing labeled tandem repeats to a genomic DNA sample was demonstrated as an effective method of quantifying the size of a DNA locus. The markers could either be fluorescence probes which could be counted via photobleaching, or gold beads which can be counted using TEM. Although single repeat lengths of 4 bp is most desirable for this approach, in this work we restricted ourselves to 8 bp repeats owing to their greater thermal stability. As the results showed, the annealing of the 8 bp repeats was not always optimal and, especially for odd numbers of repeats in the locus, the results could fluctuate around the actual size. Therefore future developments of this approach will need to be reproducible, and preferably will move

	TPOX				FGA			
Sample	Allele 8 RFU	Allele 11 RFU	PHR (%)	Drop-in alleles (RFU)	Allele 21 RFU	Allele 24 RFU	PHR (%)	Drop-in alleles (RFU)
1	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-
10	122	-	-	-	84	75	89.3	-
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
13	-	108	-	-	91	-	-	-
14	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-

Table 4. Compilation of the results of the 15 genotyping attempts without pre-amplification. The height of the peaks of the known alleles at TPOX and FGA loci are reported only if they exceed the LOD, otherwise a dash (-) indicates no peak with that size. In the case of a heterozygous locus, the PHR is calculated. As anticipated, without pre-treatment few of the samples resulted in any peaks, and there are no drop in peaks above the threshold recorded. Note that there were a total of 30 independent reactions; there is no connection between TPOX and FGA samples or data that happen to be on the same row.

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towards 4 bp repeats (eg. by using lower temperatures artificial nucleotides with higher binding affinities) to overcome the alignment problems of the 8 bp repeats. A foreseeable problem however is that the 4 bp repeats will be more susceptible to single nucleotide polymorphisms, abasic sites and other degredations. If the protocol allows repeated measurements, a series of measurements using different types and lengths of tandem repeats may provide the most robust results. In any event, even narrowing the size to a few possible numbers may be more information than can currently be gathered from many samples.

Photobleaching has an advantage over TEM of in principle being repeatable. As shown with the covalent attachment work above, it is possible to melt off annealed segments which are fluorescently labeled and re-label the DNA. Applying those lessons to this protocol will allow the DNA to repeatedly quantified, increasing confidence in the results. TEM of gold beads, on the other hand, does not facilitate the reusing the same DNA strands more than once.

3. Measuring STR length via NSET and FRET

The attraction of an NSET or FRET based quenching system for identifying DNA is the potential for obtaining fast results due to the lack of PCR in the procedure. As envisioned, an unknown DNA sample can be quickly melted, mixed with labeled primers, an immediately measured. The method will not work with LCN samples due to the low signal, but it will work with degraded DNA. In fact, as long as the flanking primer regions are sufficiently intact to permit annealing, the rest of the locus can in principle be severely degraded. This work is a significant step in that direction, demonstrating that identification of an unknown is possible in more than half the cases, but several factors are identified that negatively affect the reliability of the approach in its current form.

Quenching due to NSET is not so well understood that it was possible to develop a numerical analysis that yielded a reliable fluorophore-quencher distance. Furthermore, the data here showed consistently that single stranded DNA had a complex length dependent behavior

	Protocol	APH	LOD	peaks	heterozygotes	imbalances	% balance	PHR	Dropout
TPOX	SLLAP	683	59	43	17	11	35	0.48	17 (28%)
	5-Cycle PCR	61	53	0	0	-	-	-	60 (100%)
	None	63	42	2	0	-	-	0.70*	28 (93%)
FGA	SLLAP	155	44	38	12	5	58	0.69	22 (37%)
	5-Cycle PCR	48	45	0	0	-	-	-	60 (100%)
	None	59	37	3	1	0	100	0.72*	27 (90%)

Table 5. For the three protocols at two different loci, the average peak height (APH) was calculated from all of the peaks which rose above the limit-of-detection (LOD), regardless of whether they exceeded the analytical threshold (AT) of 75 RFUs. Also tabulated are the number of peaks at the true allele positions which exceeded the AT and the number of heterozygous loci. Loci were considered imbalanced if the smaller allele was less than 60% of the larger allele. The average peak height ratio was calculated for the SLLAP protocol by averaging the PHR of all of the heterozygous loci. *For the no pre-treatment protocol, all peaks above the LOD were considered for determining heterozygous loci, not just those above the AT. The number (percentage) of dropout is calculated as the number (percentage) of alleles that did not exceed the AT.

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with the apparent end-to-end distance (as interpreted based on quenching efficiency) repeatedly increasing, then decreasing, as the DNA length increased. Fortunately, the exact pattern of this oscillation depended on the size of the gold bead used as a quencher, so different beads yielded different signals which could be used to resolve any ambiguities. Therefore, although it was not possible to calculate the distance between the ends, it was possible to use a 'look-up table' approach to the analysis where the behavior of an unknown measured using several bead sizes was compared to the signal from all possible knowns. The known with behavior most similar to the unknown was used to identify the sample. This approach led to successful identification of samples in a blind experiment, but success rates were typically just above 50%.

In practice, the day-to-day variation in the behavior of the samples required that the known samples be measured each day in order to generate a useable look-up table. In principle this is the approach used in capillary electrophoresis, however in our case the daily measurement of a full set of controls that included all possible lengths and several gold bead sizes was a time consuming process that limited the number of bead sizes we could reasonably use to two. Given these limitations, it is remarkable that we were consistently able to determine correctly the sizes of 50% or more of the unknown samples. An improved measurement scheme using a flow through capillary system to efficiently measure the known control samples will allow us to increase the number of different bead sizes used in the experiments, which we expect will increase the frequency of correctly identifying the sample.

4. Single-sided linear pre-amplification

Overall, SLLAP-PCR has been proven to have the desired effect of increasing true allele peak heights, reducing drop-out, and increasing the number of heterozygous profiles without increasing noise, and for most samples without introducing drop-in. These results were better than a procedure in which the number of PCR cycles was increased. However, the improvement was not as significant as the advances in commercially available kits developed contemporaneously. While we decided not to continue this line of investigation, we summarize the results of the work that was done below.

While according to our calculations the peak heights were amplified by 11 fold and 2.7 fold for TPOX and FGA respectively, these numbers must be treated with caution. Clearly, only peaks which rise above the LOD (ie, the tallest) are included in the average. If the average peak height is low due to ineffective amplification, then only the largest peaks make it over the LOD, and the average peak height is calculated using a population of peaks skewed towards the largest peaks. Therefore, a direct comparison between the 11-fold and 2.7 fold amplification values likely introduces a systematic error due differences in the population of peaks being calculated.

Interestingly, although peak height amplification was locus dependent, neither drop-out nor drop-in varied much between the loci. We don't fully understand this phenomenon yet. Assuming that peak heights are an indication of the number of amplicons available at start, the amplification of the TPOX peak suggests that SLLAP-PCR increases the number of useable amplicons prior to PCR by at least 11 fold. At FGA the amplification was only 2.7 fold. Therefore, any threshold based analysis of the resulting capillary electrophoresis signal should have excluded more FGA peaks than TPOX, resulting in greater drop-out. Yet that is not what was observed. It is possible that the SLLAP protocol approached making all the DNA present

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detectable, meaning the drop-out rate after SLLAP was due entirely to the probability that there was no DNA present in the sample. However this theory is difficult to test since determining the cause of allelic drop-out is not trivial. Finally, the reduced drop-out rate resulted in an increase in the number of heterozygous loci, but the peak height imbalances that normally accompany LCN analysis were still present, with the data suggesting that the degree of imbalance depends on the average peak heights.

B. Implications for policy and practice

The potential demonstrated in this research does suggest the development in the near future of reliable techniques for the genotyping of degraded and LCN analysis. However as a basic research project designed to explore the potential of various single molecule approaches, there is little immediate impact on the practice and policy of forensic investigations, apart from the encouraging the long term storage of samples that currently cannot be analyzed effectively. It likely will not be long for the analysis of these samples is possible.

C. Implications for further research

Although the potential for DNA analysis without amplification is demonstrated here, many details will need to ironed out before any practical application can be developed.

How far can the surface attachment strategy be scaled down? Currently we are working with very large quantities of DNA, but only because the equipment we are using was designed for 25 mm wide coverslips and several hundred microliters of solution. Though we have already demonstrated that the protocol can capture and detect single DNA strands, this was done using a pristine sample containing tens of thousand of DNA strands. First, it is necessary to see if contaminants which inhibit PCR also inhibit the annealing and ligation necessary for this protocol to work. Second, we would like to establish a microfluidics setup which would allow us to flow the contents of the sample over an active spot within the field of view of the camera. The question to answer is if there are only a few strands in tens of microliters of fluid, will flowing the liquid over an active region be sufficient to cause the DNA fragments to anneal and ligate? Furthermore, such a setup would simplify the washing, rinsing, and relabeling of the surface for repeated measurements. Our current approach of heating, washing and treating the cover slips in beakers may contribute to the low number of repeat uses (3 or 4) before the increased background makes further measurements impossible.

What is the optimum configuration for annealing tandem repeats to the DNA sample? Intuitively, the annealing protocol would likely provide more robust results if we could use 4 bp repeats instead of 8 bp repeats. By using 8 bp repeats, we guarantee difficulties with samples that have an odd number of repeats. Second, especially with the larger loci, there is always the possibility that the 8 bp oligo will anneal in a position that is only 4 bp away from the flanking region, insuring that the STR region cannot be effectively filled. Both of these issues would go away with 4 bp repeats. However, it is very difficult to order a 4 bp repeat, and second, a 4 bp repeat may not have a sufficiently high affinity to anneal to the DNA strand. If the 4 bp repeats

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anneal and remain long enough to be ligated to their neighbor, then the rest of the protocol falls into place. Once ligated, they are long enough to remain annealed. The question to be answered is what are the optimum condition? Will a colder environment (which also slows the ligase) be sufficient to work with 4 bp repeats, and if not will synthetic nucleotides such as bridged nucleic acids or amino nucleic acids increase the binding affinity without disrupting the rest of the experiment? Finally, although this protocol was verified with genomic DNA, we have not yet tested how it performs in the presence of real world contaminants and inhibitors.

How reliable can fluorescence quenching experiments become? Our use of only two bead sizes was based largely on the amount of time necessary to measure the control samples, which must be done each day anew. We expect that our 50% identification success will increase if we can efficiently use more beads in the comparison. The signals we were measuring certainly were sufficiently high that the measurement time could be shortened. Our hope is that with a microfluidics setup as describe above, the control measurements could be automated, allowing a larger number of bead sizes to be included in the measurements. Again, how this protocol reacts to the presence of inhibitors needs to be explored.

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VI. Dissemination of Research Findings

Publications

Simms TM, Alhussainalali S, Antonik M. Covalent surface attachment of genomic DNA for nano-spectroscopic forensic analysis. *In preparation*.

Simms TM, Antonik M. Direct visualization of the number of STR repeats via single step photobleaching. *In preparation*.

Simms TM, Antonik M. Near surface energy transfer DNA ladder for forensic applications. *In preparation*.

Simms TM, Antonik M. Quenching of fluorescently labeled DNA using gold nanoparticles: A forensic application. *Submitted.* ChemBioChem.

Presentations

T Simms, M Antonik Single Step Photobleaching: A PCR free Alternative to Typing Low Copy Number Templates AAFS Annual Conference Feb 2015

Simms TM, Antonik M. Single Molecule Imaging: A PCR-free Mechanism for DNA profiling - Lecture. Florida International University - Miami, FL, December, 2014.

Simms TM, Antonik M. Nanometal Surface Energy Transfer (NSET): An Alternative DNA Profiling Technology - Lecture. Midwestern Association of Forensic Sciences Conference – St. Paul, Minnesota, October, 2014.

Simms TM, Antonik M. Alternative Methods to Typing Forensically Challenged Samples - Poster. Green Mountain DNA Conference - Burlington, Vermont, August, 2014.

Simms TM, Antonik M. Profiling DNA without PCR: A single molecule based approach – Lecture, OMICS 2nd International Conference on Forensic Research and Technology - Las Vegas, Nevada, October 2013.

Simms TM, LeGresley SE, Antonik M. Single molecule fluorescence: an alternative approach for the analysis of compromised forensic DNA evidence - Poster, KU Postdoctoral Association Research Day and STEM Career Fair, The University of Kansas - Lawrence, Kansas, March 2013

M Antonik, S LeGresley, J Wilt, J Ims, T Simms; Towards Genotyping Single DNA Molecules without PCR NIJ conferees meeting at AAFS, Feb 19, 2013

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Simms TM, LeGresley SE, Antonik M. TIRF: A novel non-PCR based approach to genotyping low copy number DNA - Poster, Higuchi Biosciences Center Science Talks, The University of Kansas - Lawrence, Kansas, February 2013.

M Antonik, S LeGresley, J Wilt, J Ims, T Simms; “Is DNA analysis possible without PCR” Green Mountain DNA conference Aug 1-3, 2012

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