# SINGLE MOLECULE DETECTION OF TUBERCULOSIS NUCLEIC ACID USING DARK FIELD TETHERED PARTICLE MOTION

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## ABSTRACT

Current methods for tuberculosis nucleic acid detection require amplification and labeling before detection is possible. We propose here a method for direct detection using Tethered Particle Motion: gold nanoparticles are tethered to a glass substrate by single-stranded DNA molecules consisting of the complementary sequence to the target. Detection takes place by observing a change in the motion of the nanoparticles. The particles are imaged by a dark field microscope and captured on an EMCCD camera. Single particle tracking is carried out through maximum likelihood estimation of the Poisson noise limited Gaussian image profile using a parallelized algorithm on a GPU. The method is characterized by biophysical modeling and the ability to detect nucleic acids is shown. This single molecule method is suitable for multiplexing and could form the basis of an exquisitely sensitive method of detecting the presence of nucleic acids derived from human pathogens directly from patient material.

***Index Terms*—** Biophysics, DNA, RNA detection, dark field microscopy, single particle tracking, GPU parallelization

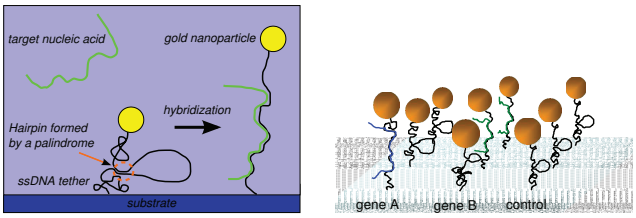
## 1. INTRODUCTION

At the moment amplification based nucleic acid assays form the most optimal (fast, sensitive) test for TB infection and are readily available in Western countries [1, 2]. Unfortunately they have not found world wide application, particularly in high burden countries, because of the requirement for dedicated DNA clean laboratories for nucleic acid amplification and/or their high cost. Efforts to simplify and automate these assays are ongoing but the required nucleic acid amplification requires specialized storage of reagents and either a dedicated lab or a complex expensive disposable device [2]. As immunological assays are unsuitable for the diagnosis of a range of infections (for example active tuberculosis) a nucleic acid based diagnostic with the sensitivity and specificity to detect nucleic acid extracted *directly* (i.e. no amplification or labeling) from patient material could revolutionize the provision of this type of diagnostics. The technology would have numerous application to other infectious diseases such as dengue, HIV, and malaria.

We propose a detection concept that is based on a technique called Tethered Particle Motion (TPM) [3], wherein we tether gold nanoparticles to a substrate using single stranded DNA molecules. The tether consists of specific DNA that matches nucleic acids to be detected. The particle-molecule system exhibits confined Brownian motion in liquid. By following the motion of the particle through the microscope, properties of the tether can be deduced. Changes in the motion of the gold nano-particle report the presence of the target nucleic acid.

## 2. DARK FIELD TETHERED PARTICLE MOTION

In Tethered Particle Motion (TPM) a reporter particle is tethered to a substrate using a (semi-) flexible polymer. The (projected) motion of the particle is followed in a microscope and captured on a CCD camera. From the position distribution of the particle the (micro-) mechanical properties of the tethering molecule can be deduced. As a quantifier of the motion




(a) Principle (b) Multiplexing

**Fig. 1**: Principle and multiplexing of the tethered particle motion method for nucleic acid detection

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of the particle we use the rms excursion, i.e. the rms distance to the anchorpoint of the DNA. Interactions with the tether can be detected by observing a change in the rms excursion. To enhance the sensitivity of the measurement the tether can be designed to have a hairpin or loop by incorporating a self-complementary sequence that can bind the ssDNA together. The target nucleic acid has a higher binding affinity to the self-complementary sequence and will open the hairpin (see figure 1(a)).

Usually in TPM the reporter particle is a micrometer sized polystyrene bead, which is quite large compared to the tethering DNA (lengths on the order of a hundred to a few thousand nanometers are commonly used). The large size of the particle restricts the conformation space of the tethering molecule resulting in an upwards, stretching force on the tether. We therefore use much smaller gold nanoparticles (with a radius of only 40 nanometers), which have the additional advantage that due to their large scattering cross section their are easily made visible using a dark field microscope [4, 5].

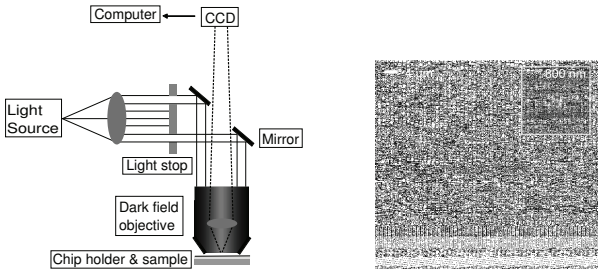
The assay has the potential for multiplexing by spotting different tether molecules on the substrate spaced sufficiently far from each other for the molecules not to overlap. This would allow in principle the development of syndromic diagnostics (targeting a range of pathogens responsible for a single clinical condition) or allowing the genetic characterization of the infection organism (for drug resistance or epidemiological markers for example). Figure 1(b) shows how the principle of the tethered particle motion method for detecting nucleic acid can be multiplexed.

### 2.1. Materials

A glass flow cell is constructed by taping two cover glasses together with two strips of double sided tape leaving a channel in between. To prevent sticking of the nanoparticle or DNA, the flow cell is first incubated with blocking buffer to passivate the glass surface. Then the surface is covered with $\alpha$-digoxigenin and subsequently incubated with DNA molecules labeled with digoxigenin at one end and biotin at the other end. Gold nanoparticles with a radius of 40 nanometers and covered with $\alpha$-biotin are added to attach to the free end of the DNA tether. Generally the tethering DNA can be either single or double stranded and has a contour length between 1000 and 5000 basepairs (300-1500 nanometers). Here we use single stranded DNA with a contour length of about 1000 nucleotides.

### 2.2. Dark field microscopy

The flow cell is placed on the specimen holder of a dark field (objective type) microscope see figure 2(a). The images are captured using a CCD camera where we typically collect 1500 to 2000 frames with an exposure of 2 to 5 milliseconds. There is a tradeoff between long and short exposure: the longer the exposure time, the more photons we can collect, however longer exposures also result in motion blur averaging of the positions and an underestimation of the size of the position distribution [6]. For following the position distribution over a longer time, we do not need high framerates. However for determining the dynamics of the particle-tether system, we use an EMCCD camera with which can readout sub-arrays with a framerate over 200 fps and has virtually no readnoise.




(a) Setup (b) Sample image

**Fig. 2**: Dark field setup: The illumination and imaging light paths are separated from each other. The sample is illuminated with a hollow cone of light and only scattered light can re-enter the imaging part of the objective, therefore objects that scatter light are brightly imaged against a dark background.

### 2.3. Single particle tracking

When using high quality (virtually aberration free) objectives in microscopy the smallest distance that can be resolved is determined by the diffraction limit $\sim \lambda/2NA$, which is on the order of 200 nanometers. Single-particle tracking can be done with a much better precision using the prior knowledge that the particles are spaced much further apart than the resolution limit. Theoretically, the fundamental limit for the localization precision of a single particle is given by $\delta x = \lambda/2\pi NA\sqrt{N}$ ($N$ is the total number of photons collected) and is on the order of only a few nanometers [7]. Our nanoparticles are of a size much smaller than the resolution and are therefore imaged as the point-spread function of the microscope objective, which can be approximated excellently as a 2D Gaussian peak [8]:

$$PSF = \frac{1}{2\pi\sigma^2} e^{-\frac{((x-\theta_x)^2+(y-\theta_y)^2)}{2\sigma^2}}$$

The location of the particle is found by maximizing the likelihood function of the Gaussian peak taking into account Poisson noise (due to the stochastic nature of the photons arriving at the detector) and background:

$$L(x,y|\theta_x\theta_y\theta_{I_0}\theta_{bg}) = \prod_k \frac{\mu_k(x,y)^{x_k} e^{-\mu_k(x,y)}}{x_k!}$$

with $\mu_k(x,y) = \theta_{I_0} PSF(x,y) + \theta_{bg}$ the expected number of arrived photons and $x_k$ the measured number of photons at

pixel $k$. This maximum likelihood estimation is implemented in a parallelized routine running on a GPU [9]. The Cramer Rao lower bound (CRLB) of the estimation is computed by analytically computing the inverse of the Fisher information matrix:

$$I(\theta)_{i,j} = -E\left[\frac{\partial^2 \ln(L(x,y|\theta))}{\partial\theta_i\theta_j}\right]$$

We determined the localization precision of our method by tracking stationary particles, bound to the substrate. We found that the positions had a standard deviation of 5 to 20 nanometers depending on exposure time and camera type. This number is somewhat larger than the fundamental limit, indicating the presence of residual motion of the stationary particles. The localization precision represents the sensitivity of detecting changes in the motion of the particles and therefore the sensitivity of detecting binding events.

### 2.4. Analysis

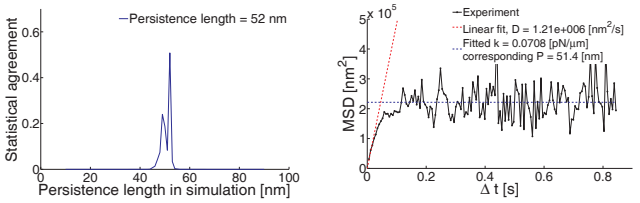
Slow drift of the microscope stage should be subtracted from the measured motion of the nanoparticles. The drift is found by smoothing the data with a gaussian kernel with $\sigma = 150$ frames (much longer than the dynamics of the motion). Subtracting the thus found drift is analogous to high pass filtering of the motion with a very small cutoff frequency. An anisotropic position distribution can arise if the particle is multiply tethered to the surface [10], therefore only particles with isotropic distributions (anisotropy $\ll 10\%$) are analyzed further. Distributions that are much too large or small compared to the expected distribution (see section 3) are discarded as well, leaving about $50\%$ of the particles for further analysis.

## 3. CHARACTERIZATION

The micro-mechanics of DNA is usually described by the worm-like chain (WLC) model, where the molecule is defined as a smooth curve with a certain persistence length i.e. the characteristic length over which directional correlation of the curve decays. When the force exerted on the molecule is sufficiently small ($< 0.1$ pN) a worm-like chain can be modeled as a Hookean spring, therefore the motion of the nanoparticle can be compared to Brownian in a harmonic potential. Thus the dynamics, or the speed/displacement of the motion is given by the diffusion properties of the particle-tether system and the position distribution is solely determined by the micro-mechanical properties of the tethering DNA.

### 3.1. Position distribution

Although analytical approaches were used to determine the position distribution for worm-like chains with constrained ends, as of yet there is no simple analytical formula for the position distribution of tethered particles. We therefore obtained simulated position distributions by sampling a large number of possible particle-tether conformations using Monte Carlo simulations. By statistically comparing the experimentally obtained position distribution to simulations, the persistence length of the DNA can be determined from the simulated distribution that has the highest statistical agreement with the experiment (see figure 3(a)) [4].




(a) Persistence length (b) Displacement plot

**Fig. 3**: (a) Persistence length of a 4899 basepair double stranded DNA molecule measured from statistically comparing the position distribution to simulated ones. (b) Mean squared displacement as a function of time difference for the same double stranded DNA molecule. Both methods obtain an approximately equal value for the persistence length.

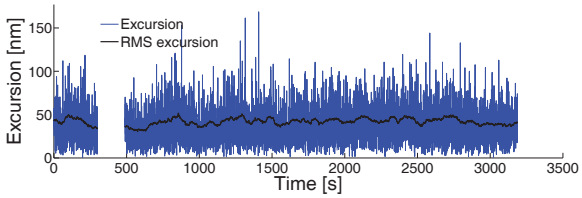
### 3.2. Dynamics

The dynamics of the particle motion can be visualized in a mean squared displacement (MSD) plot, where the displacement of the particle is plotted as a function of the time difference (see figure 3(b)). For short time differences the displacement is linear, owing to the free diffusion of the particles. The diffusion constant can be determined from the slope of the MSD: $\langle \Delta x^2 \rangle = 4D\Delta t$. For longer time differences the particle experiences a restoring force towards its center position due to the harmonic force of the tethering DNA. The MSD reaches a plateau: $\langle \Delta x^2 \rangle = 4k_BT/k$ [11], with $k = 3k_BT/2PL$ the entropic spring constant of the DNA depending on its contour length $L$ and persistence length $P$ [12]. The motion needs to be captured with a high framerate (100-200 fps depending on the length of the tether) to be able to observe the linear part of the MSD.

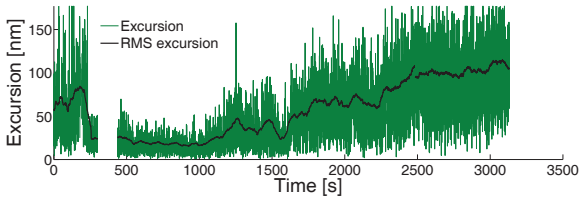
### 3.3. RNA detection

The motion of the nanoparticles is usually quantified by the rms excursion of the particle, which can be followed over long periods of time in our flow cell. Figure 4 shows the excursion of two nanoparticles, tethered by single stranded cDNA from luciferase, where after 300 seconds from the start of the measurement luciferase RNA was injected. In (a) the rms excursion stays at the same level corresponding to a single stranded tether. In (b) the rms excursion starts to increase after 900 seconds until it reaches a plateau after approximately

2300 seconds. The rms excursion there corresponds to a double stranded tether, meaning that hybridization of the RNA to the tether is not instantaneous but is a dynamic process where eventually the tether is fully hybridized.




(a) No RNA detected




(b) Successful RNA detection

**Fig. 4**: The excursion of two nanoparticles tethered to the substrate using 854 nucleotides long cDNA (single stranded) from luciferase. After 300 seconds luciferase RNA is injected. In (a) no change is detected, however in (b) the excursion slowly increases to a level corresponding to a double stranded tether, indicating that the RNA is eventually fully hybridized to the tether.

## 4. CONCLUSIONS

We have presented here a single molecule method for detection of nucleic acid directly from tuberculosis bacteria. The method consists of following the motion of a gold nanoparticle tethered to a glass substrate using a single stranded DNA molecule. The motion of the particle can be tracked very precisely by observation in a dark field microscope and applying single particle tracking to the collected images. Hybridization of nucleic acid can be detected by observing a change in the motion of the particle. We have characterized the method by interpreting the position distribution and dynamics of the particle motion and have shown successful detection of RNA using dark field tethered particle motion. The next step is to determine the sensitivity and specificity of the detection. In further work, the method can be multiplexed for the detection of multiple nucleic acid sequences and has the potential to dramatically improve the near patient provision of DNA/RNA based diagnostics.

## 5. REFERENCES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