**Introduction:**
Protein-DNA and protein-RNA interactions are among the most interesting interactions that can be studied. These include mechanisms that are crucial for cellular activities, including DNA polymerases, packaging proteins (such as histones) etc. In order to understand those interactions properly, Single Molecule Detection methods should be used. Here we present an improvement to one of the common methods, Tethered Particle Motion (TPM), by applying it in three dimensions (3D). The method allows to track the studied molecule by following the position of it in 3D with high precision of few nanometers. Therefore, more biophysical information can be extracted.

**Tethered Particle Motion (TPM):**
A small bead is linked to one end of the sample molecule (DNA or RNA), while the other end is linked to the glass surface. The bead diffuses in a physiological solution, and its position is detected by an optical microscope. By analyzing the statistics of $r(t)$, one can find the molecular characteristics, such as persistence length and DNA spring constant. Measurements of the interacting system enables the detection of the characteristics changes.

**Total Internal Reflection Microscopy (TIRM):**
In order to find the distance from the surface (along the z dimension), we use TIRM. When total internal reflection is generated (figure below), an electromagnetic field is generated in the other side of the surface called evanescent field. The power of this field decreases exponentially with distance. Therefore, by measuring the scattered intensity from the bead, one can find the distance from the surface.

**Experimental Setup:**
We detect scattered light from gold nano-bead (typical diameter of 50 nm). This method has several advantages:
- The small size of the bead does not affect the DNA motion.
- The scattering from metal bead has high efficiency (Mie scattering), and therefore the signal is bright and easy to detect. This results in higher accuracy in calculating the bead position and better statistical information.
- In contrast to fluorescent methods, there is no photobleaching, and high signal intensity is detected for a long time. It is therefore possible to follow slow processes or measure the same reaction number of times.

One end of the DNA molecule is linked to the surface via digoxigenin - anti digoxigenin, and the other end is linked to the nano-bead via biotin - anti biotin.

The sample is placed in a chamber, and the biochemicals (DNA, bead etc.) are injected to the chamber through tubes that are connected to it. To achieve the evanescent field, we use 658 nm diode laser and an equilateral prism. The scattered light is detected with a CCD camera, and image analysis provides both the $x$, $y$ and $z$ coordinates.

**Data analysis:**
The distribution function of the bead position is the distribution of “random-walk”, and by fitting the measured data to the theoretical distribution function, the persistence length of the DNA is extracted. The finite exposure time of the camera implies an error (the variation of the measured data seemed narrower than the actual one). We therefore use a correction function in order to calculate the persistence length.

For 1-dimension ($x$ or $y$ axes), the distribution function is a Gaussian:

$$P(r) = \frac{1}{\sqrt{2\pi L^2 I_p}} e^{-\frac{r^2}{4 L^2 I_p}}$$

and for 2-dimensions, Rayleigh distribution:

$$P(r) = \frac{1}{\sqrt{2\pi 4 L^2 I_p}} e^{-\frac{r^2}{4 L^2 I_p}}$$

Where $L$ is the nominal length of the DNA (~1µm in our case), and $I_p$ is the persistence length.

The distribution in the $z$-axis is different and more complex than the distribution in the $x$ or $y$ axes, because of the constrained volume of the surface. The intensity of the evanescent field decreases exponentially with height, reaching maximum at the surface, therefore, the position of the bead in the $z$-axis is proportional to the logarithm of the light intensity. The calibration of the penetration depth of the evanescent field done by fitting the distribution of $-\ln(I)$ to the expected distribution of the $z$-axis using the parameter that found from the $x$-$y$ distribution.

**Results:**
As one can see in the graphs below (C), measuring with TIR does not change the radial distribution of the bead. The calculated persistence length is ~50nm for both experiments (using darkfield illumination first, and using TIR illumination later), in agreement with literature.

Although the light intensity is not uniform even with the darkfield illumination (due to blinking effect etc.), it is easy to see (D) that the intensity distribution is much narrower then in the TIR illumination. This confirms the validity of the TIR data.