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### **Tracking Protein-coated Particles in 3D.<sup>1</sup>**

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The utilization of 2-photon microscopy in the field of Cell Biology is of increasing importance because it allows imaging of living cells, including those systems where UV imaging is not possible due to photobleaching or photodamage limitations. We propose a novel approach using 2-photon excitation based on the use of a scanner to produce an effective “intensity trap”. As the particle moves in this trap (note that there is no force applied on the particle at the power level we are using for particle detection), the detection system continuously calculates the position of the particle in the trap. As the position of the particle is calculated with respect to the trap, the scanner position is moved to minimize the “modulation” of the light intensity in the trap. In practice, we set the scanner to perform an orbit around the particle in about 1 millisecond. The sampling rate is chosen such that many points (32 or 64) are acquired during the orbit. An FFT (Fast Fourier Transform) is performed on the points acquired during one orbit or after a series of orbits. The DC, AC and phase of the first harmonic of the FFT are calculated. The value of the modulation varies monotonically as the distance of the particle from the center of the orbit is increased so that for every value of the modulation we can estimate the value of the distance of the particle from the center of the orbit. The phase of the first harmonic gives the angular position of the particle with respect to the scanner zero phase which is known relative to the lab coordinates. The effective bandwidth of the tracking system depends on the maximum frequency for sinusoidal oscillation of the scanner, which is about 5 kHz for our galvano-scanner and on the number of photons needed for detecting the particle against the noise. Of course, there are other important considerations. First, if the motion of the particle is too fast such that after one orbit the particle moves too far from the new position calculated based on the previous orbit, tracking is lost since the feedback mechanism is too slow. Therefore, single molecules, which in water would move across the PSF in about 0.1 ms, cannot be tracked. We need at least a macromolecule the size of a large protein (100kD) or relatively high viscosity to increase the time a fluorescent particle can be observed in the PSF. The second consideration, perhaps the most important, is that the particle should not bleach during the length of the tracking. This is not a problem for particles made of many fluorophores, such as polystyrene fluorescent beads, which are also relatively large. Surprisingly, for relatively large particles such as viruses, photobleaching did not occur.

<sup>1</sup>In collaboration with Valeria Levim, Univeristy of Illinois at Urbana-Champaign.