

Two-photon fluorescence excitation in detection of biomolecules

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Synopsis

Two-photon fluorescence excitation has been found as a very powerful method for enhancing the sensitivity and resolution in far field light microscopy. Two-photon fluorescence excitation also provides a substantially background free detection on single molecule level. It allows direct monitoring of formation of labeled biomolecule complexes in solution. Because the emission of two photon excitation is a quadratic process with respect to illumination intensity, only the fluorescence that is formed in the clearly restricted 3-dimensional vicinity of the focal point is excited. We have developed an assay concept that is able to distinguish optically between the signal emitted from a microparticle in the focal point of the laser beam, and the signal emitted from the surrounding free labeled reagent. Moreover, the free labels outside the focal volume do not contribute any significant signal. This means that the assay is separation free. The method based on two-photon fluorescence excitation makes possible fast single step and separation free immunoassays, for example, for whole blood samples. Since the method allows a separation free assay in very small volumes, the method is very useful for high throughput screening assays. Consequently we believe that two-photon fluorescence excitation will make an remarkable impact as a research tool and a routine method in many fields of analysis.

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Synopsis

We have developed an assay concept and an instrument for monitoring bioaffinity reactions in micro volumes. The concept is based on use of microparticles as solid phase carriers for bioaffinity reaction. The illumination of bioaffinity fluorescence signal is based on two-photon fluorescence excitation. The emission of two-photon excitation is a quadratic process with respect to illumination intensity, thus only the fluorescence that is formed in the clearly restricted 3-dimensional vicinity of the focal point is excited. In our assay concept it is possible to distinguish optically between the signal emitted from a microparticle in the focal point of the laser beam, and the signal emitted from the surrounding free labeled reagent. Moreover, the free labels outside the focal volume do not contribute any significant signal. This means that the assay is separation free.

Instrument and assay

The optical schematic of the instrument, TopDoc, is shown in **Fig 1**. The beam of the passively q-switched microchip Nd:YAG-laser at 1064 nm is focused tightly into a *cuvette*. The generated fluorescence in range 540-600 nm is measured with a photomultiplier tube (PMT) in coincidence with back scattering signal of the microparticles in the confocal *particle detector*. As the scattering signal appears, the *scanner* unit is stopped, and the particles are actively trapped into the focus of laser beam to prolong the measurement period (**Fig 2**).

Both immunometric and competitive assays have been tested. The model assay is immunometric, utilizing 3.1 μm amino-modified polystyrene microspheres as solid phase carriers. Mouse anti-AFP IgG fragments are covalently coupled to the microspheres. As a tracer mouse anti-AFP IgG fragments were labeled with dipyrrometheneboron difluoride dye having single photon excitation maximum at 530 nm. Analyte and reagent were mixed simultaneously and incubated in prior to measurement in the same reaction cuvette.

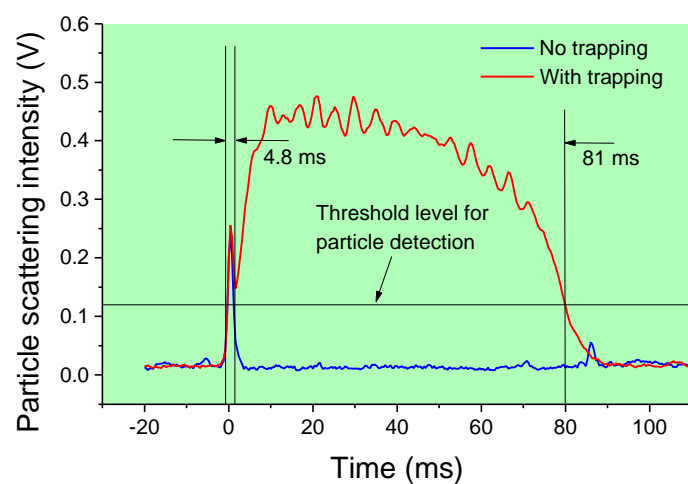


Figure 2. Effect of active trapping.

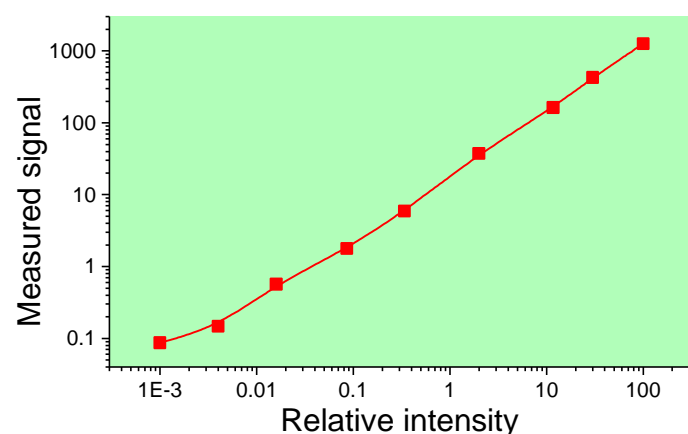


Figure 3. Measured instrument response to standardized microparticles (Linear Flow Green, Molecular Probes).

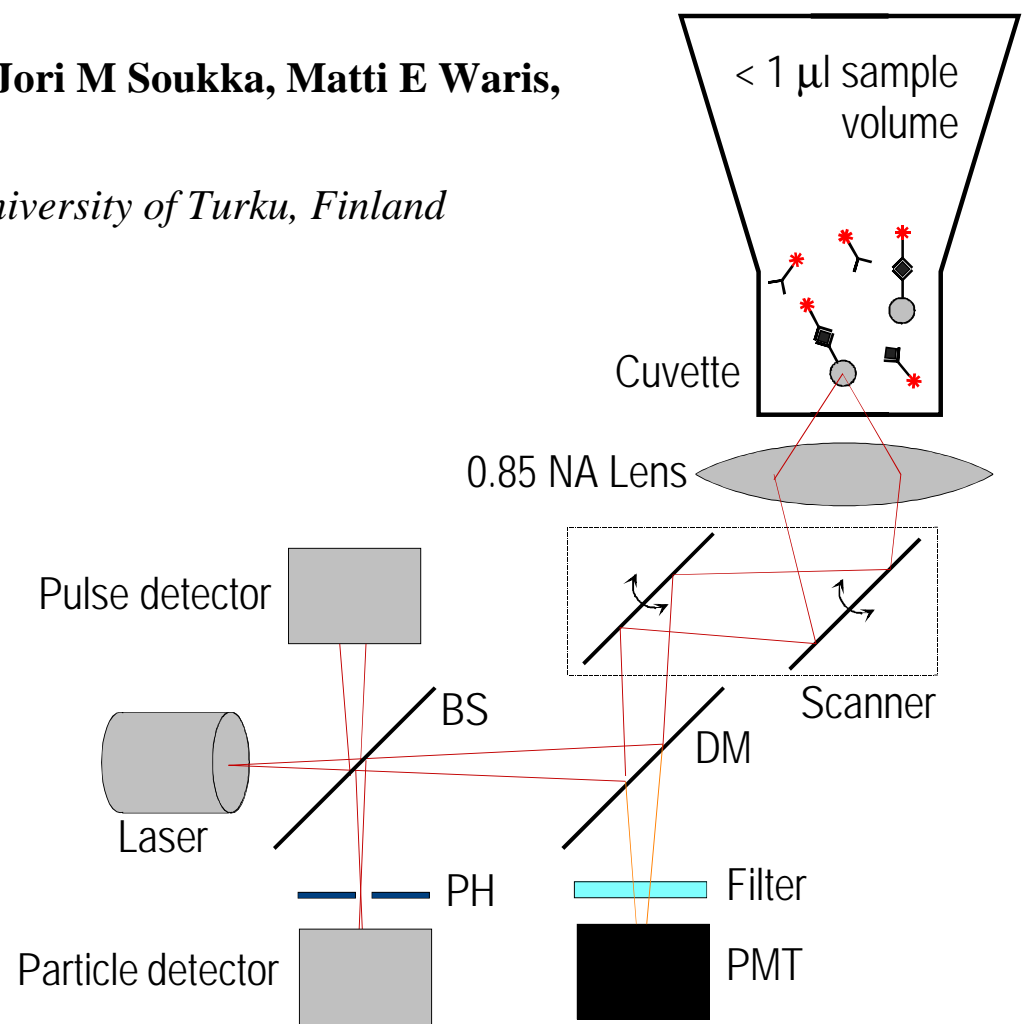


Figure 1 TopDoc microfluorometer optical schematics.

Results

The linear instrument response covers a range of 5 o.o.m. (**Fig 3**). The standard curve of the AFP-assay is shown in **Fig 4**. Each point of the curve comprises of an average of around 100 microparticles. The CV at the blank level was as good as 3%. The sensitivity of 0.2 ng/ml was obtained by relating the assay response to the usual 3σ (standard deviation) level of the blank sample signal. The assay was done in 25 μl volume but can be directly scaled to smaller volumes, at least from the instrumental point of view. We obtained identical signals by measuring 1 μl volumes.

Conclusion

The method based on two-photon fluorescence excitation makes possible fast single step and separation free immunoassays, for example, for whole blood samples. Since the method allows a separation free assay in very small volumes, i.e. less than 1 μl , the method is very useful for high throughput screening assays. Consequently we believe that two-photon fluorescence excitation will make a remarkable impact as a research tool and a routine method in many fields of analysis.

Acknowledgments

SRL, Inc., Tokyo, Japan

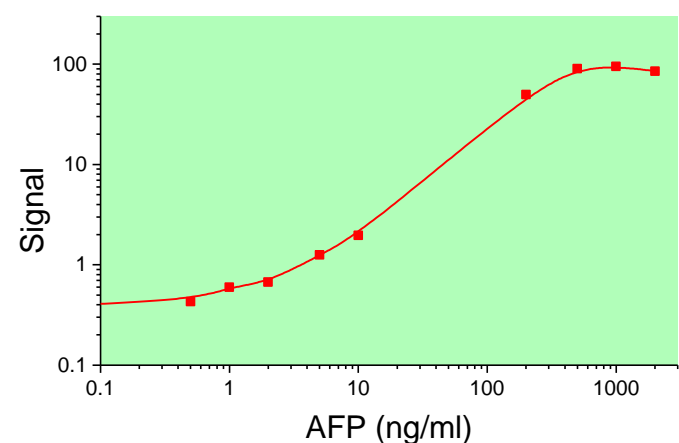


Figure 4. Measured AFP-assay standard curve.