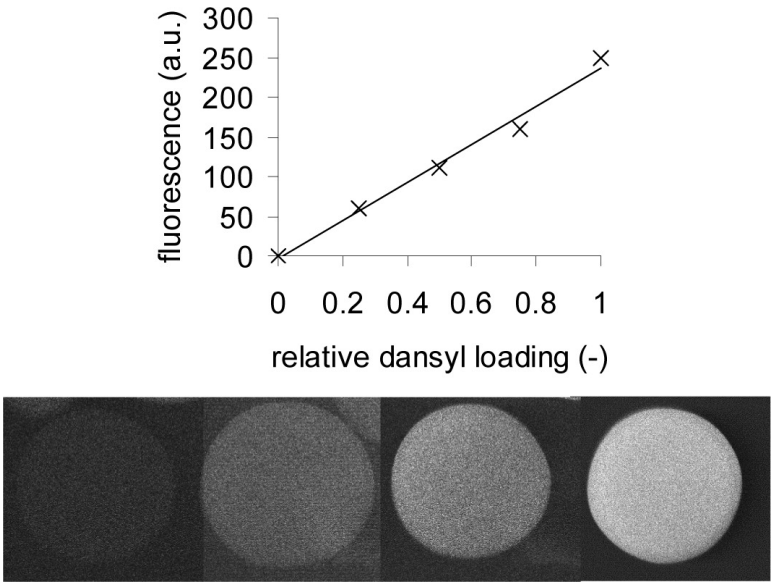
JOURNAL OF combinatorial CHEMISTRY

Report

Two-Photon Microscopy to Spatially Resolve and Quantify Fluorophores in Single-Bead Chemistry

Rein V. Ulijn, Ian Brazendale, Graham Margetts, Sabine L. Flitsch, Gail McConnell, John Girkin, and Peter J. Halling

J. Comb. Chem., **2003**, 5 (3), 215-217• DOI: 10.1021/cc030024c • Publication Date (Web): 09 April 2003



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 3 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

Two-Photon Microscopy to Spatially Resolve and Quantify Fluorophores in Single-Bead Chemistry

Rein V. Ulijn,*,† Ian Brazendale,⊥ Graham Margetts,⊥ Sabine L. Flitsch,† Gail McConnell,§ John Girkin,§ and Peter J. Halling‡

School of Chemistry, King's Buildings, The University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, U.K., Department of Chemistry, Thomas Graham Building, The University of Strathclyde, 295 Cathedral Street, Glasgow G1 1XL, U.K., Centre for Biophotonics, Strathclyde Institute for Biomedical Sciences, University of Strathclyde, 27 Taylor Street, Glasgow G4 0NR, U.K., and Polymer Laboratories Ltd., Essex Road, Church Stretton, Shropshire, SY6 6AX, U.K.

Received March 12, 2003

Solid-phase chemistry on polymer beads is widely used in the production and biological screening of combinatorial libraries of compounds.[1] Since a lot of analytical methods used in solution chemistry cannot be applied to solid-phase chemistry, monitoring chemical processes on beads has been difficult. To understand solid-phase chemistry, it is desirable to describe the distribution of chemical groups within the solid-phase supports. Previous studies have described the use of autoradiography,[2] scanning secondary ion mass spectrometry,[3] fluorescence,[4] and several microscopy methods, such as confocal fluorescence,[5] confocal Raman,[6,5d] infrared,[7] and more recently, two-photon microscopy (TPM).[8] However, the spatial quantification of the chemical groups present has not been described before. Here, we show that TPM allows for the direct quantification of fluorophore distributions within single $PEGA_{1900}$ (poly(ethylene glycol)acrylamide) beads.

In TPM,[9] the sample is irradiated with a laser with a wavelength approximately twice that of the normal excitation wavelength of the fluorophore. As a result, excitation can occur only when two photons are absorbed simultaneously. Such two-photon events occur at a very high photon density that is reached only at the focal point of the laser beam. Hence, the fluorescence detected originates only in the part of the sample that is in focus. Away from the focal point, there is essentially no absorption of the exciting beam, because there is no chromophore able to absorb single photons of this wavelength. Hence, TPM avoids the artifacts caused by excitation attenuation due to absorption that have been described for confocal fluorescence microscopy.[7] In addition, the risk of photodamage to the sample is reduced by application of a pulsed laser permitting recovery of the fluorophore.

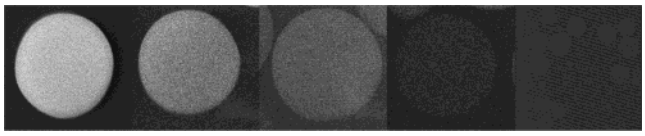
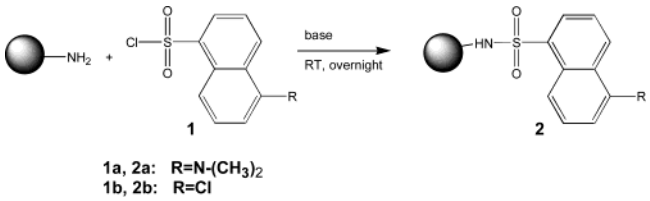


Figure 1. Fluorescence images of PEGA1900 beads of 100-μm size treated with different ratios of dansyl/nonfluorescent analogue.[12] From left to right, these ratios are 1, 0.75, 0.50, 0.25, and 0.0.[13] The total amine loading of the resin is 200 μmol/g.[14]

Scheme 1. Varying Ratios of Dansyl Chloride (**1a**) and a Nonfluorescent Dansyl Analogue (**1b**) Are Coupled to $PEGA_{1900}-NH_2$



Our first aim was to establish whether we could use TPM to measure the distribution of fluorescent groups within $PEGA_{1900}-NH_2$ beads. To this end, these beads were treated with an excess of dansyl chloride under reaction conditions that should result in uniform dansylation of all amino groups on the polymer. The beads were then thoroughly washed to remove any nonspecifically bound dansyl and investigated using TPM.[10] Since the excitation maximum for dansyl derivatives is reported to be $\lambda = 340$ nm, a laser of $\lambda = 770$ nm wavelength was used.[11] The resulting fluorescence image of the largest cross section of a single bead (Figure 1, left image) reveals a uniform distribution of functionality within the beads in the lateral direction, as also observed recently for polystyrene[5,6] and TentaGel.[8]

Next, we moved on to establish whether the fluorophore concentration within single beads could be quantified directly. Predetermined concentrations of fluorophore were obtained by reacting $PEGA_{1900}-NH_2$ with mixtures in different ratios of dansyl chloride **1a** and a nonfluorescent analogue, **1b**, to form **2a/2b** mixtures in corresponding ratios (Scheme 1, 1a).[12] Both **1a** and **1b** are expected to be equally reactive because they differ only in a part of the molecule that is remote from the reacting sulfonyl chloride group. Figure 1 (bottom row) shows the cross-sectional images that were obtained using TPM from the largest cross section (the middle) of 100-μm-diameter beads with 100, 75, 50, 25, and 0% dansyl loading.

By focusing the laser beam at different depths within the sample, optical sections at discrete depth intervals within the sample can be obtained (Figure 2). By adding these optical sections together, a three-dimensional image of the sample can be constructed.

Figure 2 shows that at all depths, the fluorescence emission is homogeneously distributed in the lateral direction; however, upon increasing focal depth, the fluorescence emission signal is attenuated.

† The University of Edinburgh.
‡ Department of Chemistry, The University of Strathclyde.
§ Strathclyde Institute for Biomedical Sciences, University of Strathclyde.
⊥ Polymer Laboratories Ltd.

10.1021/cc030024c CCC: $25.00
Published on Web 04/09/2003

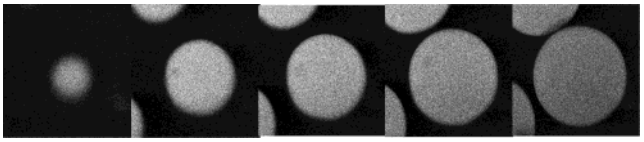


Figure 2. TPM images of several optical slices of a 150-μm $PEGA_{1900}$ bead loaded with dansyl chloride. The images reveal that the distribution of chemical groups is homogeneous in the lateral direction. Upon increasing the focal depth, fluorescence signal attenuation is observed.

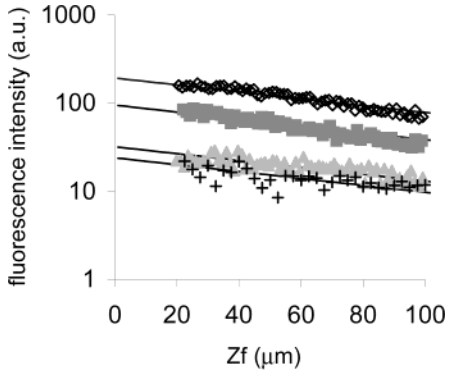


Figure 3. Measured TPM fluorescence as a function of focal depth for 150-μm $PEGA_{1900}$ with different dansyl loadings beads. Diamonds represent 100% dansyl; squares are 75%; triangles, 50%; and crosses, 25%. Note that the intercepts are not in the expected proportions. This is caused by inhomogeneities within populations of beads, as discussed further.

Contributions to weakening the signal with increasing depth may come from aberration, scattering, and absorption. These may reduce both the number of excitation photons reaching the focus area and the number of fluorescent photons collected by the objective. Dunn et al have shown that the measured two-photon excited signal decays exponentially with increasing focal depth for small (<1 μm) fluorescent spheres embedded in turbid gels.[12] The resulting detector signal as a function of focal depth $I(z_f)$ was thus fitted according to

$$I(z_f) = I_0 \exp(-z_f\alpha) \quad (1)$$

where z_f is the focal depth from the top surface of the bead, I_0 is the fluorescence intensity at $z_f = 0$, and α is the attenuation parameter. Figure 3 shows the average fluorescence intensity obtained for each section at increasing focal depths for $PEGA_{1900}$ beads with different dansyl loadings. In each case, I_0 was obtained by extrapolating to zero focal depth. The parallel lines indicate that the observed fluorescence decay could be described well with $\alpha = 0.009\ \mu m^{-1}$. It might seem remarkable that increasing the focal depth attenuates fluorescence while it is homogeneous in a lateral direction, because the thickness of the bead transversed will vary depending on whether the edge or in the center of the bead is imaged. It must be that attenuation depends on the path length from the glass surface to the focal point, whether this path is entirely through the bead (central pixels) or partly through the surrounding water (toward the edge). Similar observations were described by Bradley et al.[5d] The bead must have optical properties similar to the surrounding liquid, probably because the interior of the bead is >90% water.

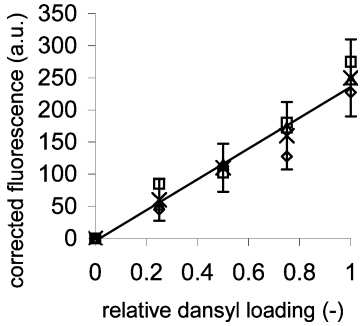


Figure 4. The relative fluorescence intensity obtained from pixel intensity data is plotted against the dansyl loading of $PEGA_{1900}$ beads of different sizes.[15] Diamonds represent 100 μm beads, squares are 150 μm, and triangles are 250 μm. Regression line, $y = 0.96x - 0.016$ with $R^2 = 0.99$. All bead sizes are normalized against a single maximum intensity.

Optical effects, thus, clearly depend on the optical path length that the photons need to travel through the sample. Hence, detrimental signal attenuation is expected to be more important when larger beads are studied.

To investigate such effects, different sizes (100, 150, and 250 μm) of $PEGA_{1900}-NH_2$ beads were obtained by sieving, with each fraction having a standard deviation of <10% from the average value. This size range covers the range of commonly used bead sizes in combinatorial chemistry. Fractions were derivatized as described above with varying ratios of **1a**/**1b**, and TPM measurements of the largest cross sections of single beads were compared. For these, the signal attenuation was corrected for using eq 1 with $\alpha = 0.009\ \mu m^{-1}$ and the radius of the bead under investigation. Figure 4 shows a plot of observed fluorescence versus the fluorophore loading in each bead. Different labels indicate different bead sizes.

When plotting the fluorescence intensity of differently sized beads against the loading of fluorophores, a linear relationship was observed with a slope of 0.96 and a correlation coefficient of $R^2 = 0.99$ (Figure 4). This observation indicates that the corrected fluorescence intensity is proportional to fluorophore loading, regardless of bead size.

From the standard deviations in Figure 4, it follows that variation between individual beads was significant, even though some obvious extremes (very dark or very bright beads) were excluded from these measurements. Similar variations were observed for all sets of beads analyzed. Hence, the conclusion is that individual $PEGA_{1900}$ beads in a sample are significantly different in their loading of reactive groups.

In summary, we have shown for the first time that two-photon microscopy can be used for the quantification of relative fluorophore concentrations within $PEGA_{1900}$ beads. Within the common size and loading range of beads used in combinatorial chemistry, the two-photon method gives reliable quantification of fluorophores present. Attenuation effects can be corrected for by taking into account an exponential drop in fluorescence with depth. Individual $PEGA_{1900}$ beads were found to have a homogeneous distribution of chemical groups within each bead.

Acknowledgment. The authors gratefully acknowledge financial support from the EC, SHEFC, BBSRC, and the Wellcome Trust.

References and Notes

(1) (a) Yan, B. *Curr. Opin. Chem. Biol.* **2002**, *6*, 328. (b) Guillier, F.; Orain, D.; Bradley, M. *Chem. Rev.* **2000**, *100*, 2091.
(2) Sarin, V. K.; Kent, S. B. H.; Merrifield, R. B. *J. Am. Chem. Soc.* **1980**, *102*, 5463.
(3) Roscoe, S. B.; Frechet, J. M. J.; Walzer, J. F.; Dias, A. J. *Science* **1998**, *280*, 270.
(4) Scott, R. H.; Balasubramanian, S. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1567.
(5) (a) Egner, B. J.; Rana, S.; Smith, H.; Bouloc, N.; Frey, J. G.; Brocklesby, W. S.; Bradley, M. *Chem. Commun.* **1997**, *8*, 735. (b) McAlpine, S. R.; Lindsley, C. W.; Hodges, J. C.; Leonard, D. M.; Filzen, G. F. *J. Comb. Chem.* **2001**, *3*, 1. (c) McAlpine, S. R.; Schreiber, S. L. *Chem.-Eur. J.* **1999**, *5*, 3528. (d) Kress, J.; Zanaletti, R.; Rose, A.; Frey, J. G.; Brocklesby, W. S.; Ladlow, M.; Bradley, M. *J. Comb. Chem.* **2003**, *5*, 28.
(6) Kress, J.; Rose, A.; Frey, J. G.; Brocklesby, W. S.; Ladlow, M.; Mellor, G. W.; Bradley, M. *Chem.-Eur. J.* **2001**, *7*, 3880.
(7) Rademann, J.; Barth, M.; Brock, R.; Egelhaaf, H.-J.; Jung, G. *Chem.-Eur. J.* **2001**, *7*, 3884.
(8) Farrer, R. A.; Copeland, G. T.; Previate, M. J. R.; Okamoto, M. M.; Miller, S. J.; Fourkas, J. T. *J. Am. Chem. Soc.* **2002**, *9*, 1994.
(9) Denk, W.; Strickler J. H.; Webb, W. *Science* **1990**, *248*, 73−76.
(10) We used a standard commercial multiphoton system (Bio-Rad MRC1024, Coherent Mira 900) coupled to a Nikon TE300 microscope. Images were acquired using a Nikon 20×/0.75 NA objective lens. Optical sections were obtained by focusing the laser beam at different depths within the sample, and by superimposing these optical sections, a three-dimensional image of the sample was constructed. With the conservative average power levels used (approximately 20 mW reaching the sample) and within the time frame of our experiment (90 s for complete optical sectioning of the large diameter beads), no photobleaching was observed.
(11) We chose $\lambda = 770$ nm to excite the fluorophore, because this offers good overlap with the absorption band of dansyl at relatively high average power.
(12) Dunn, A. K.; Wallace, V. P.; Coleno, M.; Berns, M. W.; Tromberg, B. J. *Appl. Opt.* **2000**, *39*, 1194.
(13) To 10 mg of PEGA1900−NH_2 beads was added 1 mL 80 mM aqueous $Na(CO_3)_2$ solution and 1 mL of a 5 mM solution of different ratios of **1a** and **1b**. The resulting solution was vortexed for 1 min and incubated on a blood rotator at RT overnight.
(14) Loading was tested by reacting solid-phase amines with Fmoc-glycine followed by Fmoc quantification using 20% piperidine in DMF and analysis at 290 nm.
(15) For each bead size and loading beads, at least 5 beads were imaged at random. Extremely bright or dark images were left out of the analysis at this point.

CC030024C