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Performance of Fluorescent Labels in Sedimentation Bead Arrays—A Comparison Study

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Abstract An extensive study is described to identify the most suitable fluorescent label in magnetic microsphere sedimentation arrays. The investigated fluorescent labels, commonly used in multiplex analysis, include organic dyes, (fluorescein, Alexa488, Cy5) fluorescent proteins (R-Phycoerythrin, Allophycocyanine, PBXL-3) polymer nanoparticles (FluoSpheres, PD-Pt) and semiconductor nanocrystals (Quantum dots). DNA hybridization assays on magnetic microspheres were applied as model systems to reveal label performance. The fluorescent labels were characterized under optimized conditions regarding signal intensity, non-specific binding and photo-stability. The advantages and drawbacks of individual labels are discussed. The limit of detection and dynamic ranges are determined to compare the performance of selected labels. Detection limits of 2×10^{-10} mol/L are found for the determination of oligonucleotides using PBXl-3 as label, which is comparable with typical flow cytometer systems. The results and protocols are highly valuable for any type of bead based assays and can be easily transferred.

Keywords Multiplex analysis · Suspension arrays · Fluorescent labels · Fluorescence imaging · Magnetic microspheres · Bead arrays

Introduction

Fluorescence is the most wide spread means of detection in multiplexed bioassays including planar array technologies and suspension arrays [1–4]. Multiplexed bead arrays offer advantages in handling and higher flexibility and have become an important tool in many fields of bioanalytics. In a previous report we introduced a suspension array concept based on the sedimentation of magnetic microspheres to the bottom of a reaction vessel and time-resolved imaging for array read-out [5, 6]. In this paper we investigate the suitability and performance of various fluorescent labels for this assay platform.

Numerous fluorescent labels are available and have been applied to biochips and bead based assay formats. In a first approach, the spectral compatibility of the label with the measurement system, the absorption coefficient, and the fluorescence quantum yield are important criteria for the selection of labels with regard to limit of detection and dynamic range. However, also other critical factors determine the suitability of a label in a specific application. Non-specific binding of labels to immobilised capture probes and the substrate may negatively influence detection limits and increase background noise. Photodegradation during assay preparation and measurement also needs consideration.

In this work promising label candidates are screened to reveal the most suitable for the application in microsphere sedimentation arrays. Well established fluorescent organic dye labels (fluorescein, Alexa488 and Cy5) and commercially available high-performance labels including semiconductor nanocrystals (quantum dots), phycobiliproteins, phycobilisomes and luminescent nanoparticles were tested. Besides labels applied as conjugates the double strand specific nucleic acid stain SYBRgreen I was applied. Fluorescent proteins are attractive due to their high extinction coefficients to enhance the sensitivity in microsphere sedimentation arrays. Phycobiliproteins, Allophycocyanin (APC) and R-phycoerythrin (R-PE) are extensively used as labels in diagnostic and high-throughput screening

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bead based assays [7]. Phycobilisomes (PBXL-3) are large complex assemblies of phycobiliproteins [8]. Phycobilisomes improved assay sensitivity at least fivefold when applied for DNA detection in microarrays [9] or flow cytometric immunoassays [10].

Fluorescently doped polymer nano-particles are of increasing interest as labels in microarray applications [3]. Accumulation of fluorophores in the nanobeads leads to higher fluorescence signals. Additionally, fluorophores are structurally rigidized and shielded from potential quenchers in solution, often resulting in increased quantum yields. On the other hand, the hydrophobic nature of the polymer, which is polystyrene in most cases, is the driving force of non-specific binding of the beads to surfaces. The resulting high blank signals are mentioned in the literature [11] and represent a major obstacle when applying this kind of label.

Fluorescent semiconductor nanocrystals, also called quantum dots (QDs), have attracted a lot attention in the recent years. They exhibit high extinction coefficients over a long range of wavelengths and emit light in narrow ands. Hence, they are another promising alternative in biological labelling and detection [12, 13].

In the work presented here, hybridisation assays were applied as model systems for characterisation of label performance. Extensive studies are carried out to identify the most suitable fluorescent label in magnetic microsphere sedimentation arrays. A series of fluorescent labels is characterised under optimised conditions regarding signal intensity, non-specific binding and photostability. Based on direct comparison, the advantages and drawbacks of individual labels are discussed and calibration experiments are carried out to compare performance of selected labels. The findings and protocols are highly valuable for other type of bead based assay and can be easily transferred.

Materials and methods

Oligonucleotide coupling

Carboxylated magnetic polystyrene microspheres of 7 μm diameter were obtained from micromod (Rostock, Germany). 5′-aminoC6 oligonucleotide LeuPN01 (5′-tac aag aat ccc aaa ctc acc ag-3′) was obtained from biomers.net (Ulm, Germany). For coupling, 5 μL of microbeads (50 mg/ml) are transferred to a 1.5 ml centrifuge tube and washed by magnetic separation with ethanol and 0.1 mol/L 2-morpholinethansulfonic acid buffer (MES, pH 5.3). After resuspending in 0.5 μL MES buffer, 10 μL of LeuPN01 (100 pmol/μl) and 2 mg N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDAC) are added to the suspension. Another 2 mg EDAC is added after 2 h of gentle shaking, and the suspension is shaken overnight. Subsequently, the oligonucleotide-coated beads are washed magnetically twice each with MES buffer and water and resuspended in 500 μl water as stock suspension.

DNA hybridisation assays

Oligonucleotides labelled with biotin, fluorescein, Alexa488, Cy5 and unlabelled oligonucleotides were purchased from biomers.net. Streptavidin-labelled Quantum-dots QD525, QD655 and QD705 were purchased from Quantum Dot Corporation (Hayward, CA, USA), streptavidin-labelled PBXL-3 from Martek Biosciences (Columbia, MD, USA), streptavidin-labelled yellow-green Fluospheres 505/515 were obtained from Molecular Probes (probes.invitrogen.com, Eugene, OR, USA) and streptavidin-labelled PD-Pt-nanoparticles from Active Motive Chromeon (Regensburg, Germany).

Analyte solutions contain oligonucleotides complementary to LeuPN01 at selected concentrations or none for blank samples. Assays are carried out by adding 1 μl of oligonucleotide-coupled bead stock solution to 100 μL of analyte solution, buffered by TES ((Trishydroxymethyl)-aminomethane, 0.1 mol/L, pH 8.0, 10 mM EDTA, 50 mM NaCl) in a 384-well PS microplate. The wells are sealed with microplate seal Fasson S695, gently mixed at 70 °C for 10 min and then gently mixed at room temperature for 20 min for hybridisation. Subsequently, the suspensions are transferred to a 384-well glass bottom microplate for washing and measurement. The beads are sedimented magnetically, the supernatant removed and the beads washed twice with 100 μl TES.

Subsequent to washing, directly labelled assays (Fluorescein, Alexa488 and Cy5) are resuspended in 100 μl TES and sedimented magnetically for measurement.

Unlabelled assays are resuspended in 100 μL SYBR-green staining solution (SYBRgreen stock solution in DMSO, diluted 1:10,000 in one aliquot TES and nine aliquots water), gently mixed at room temperature for 10 min for DNA staining, washed twice with TES, resuspended in 100 μl TES and sedimented magnetically for measurement.

Biotin-labelled assays are resuspended in 100 μL phosphate buffer (PB, 0.05 mol/L, pH 7.35). 3 μL of streptavidin-conjugated labels (PBXL-3, APC, R-PE, Fluospheres505/515, QD or PD-Pt respectively) are pipetted to the suspension. The suspensions are gently shaken at room temperature for 1 h to incubate streptavidin–biotin-binding. The beads are washed twice with PB containing 1% BSA (Albumin Fraction V, Carl Roth GmbH, Karlsruhe, Germany). Subsequently, beads are resuspended in 100 μl PB and sedimented magnetically for detection.

Fluorescence microscopy and imaging

All images are taken by an inverted epi-fluorescence microscope Axiovert 25 CFL (Zeiss, Gottingen, Germany) using 40× objective. Four filter sets (Tables 1 and 2) are configured to match the spectral properties of the fluorescent labels, using filters from Zeiss (Göttingen, Germany), Omega Optical (Brattleboro, VT, USA), Schott (Mainz, Germany) and Chroma (Rockingham, VT, USA).

A 50 W mercury arc lamp (HBO) is used with the 405/610 filter and a 180 W xenon arc lamp (XBO) from Linos Photonics (Gottingen, Germany) with the 475/515, 535/590 and 640/690 filters.

For assay evaluation, sections of the microplate bottom are chosen and focused in brightfield dia-illumination mode at low light intensities to reduce photobleaching. Brightfield images are taken for bead localisation. Subsequently, fluorescence images of the same section are acquired for DNA quantification.

For measurement of photobleaching, freshly prepared samples with positive oligonucleotide signal were continuously illuminated with corresponding arc lamp and filter set, while taking images in intervals of 10 s.

Image evaluation

Images were evaluated by a self-written Matlab-Script. In brief, microspheres are localised by finding local intensity maxima in the brightfield images. Subsequently, localisation artefacts are filtered out by calculating statistical central moments of zeroth and second order. Limits for these moments are set by identifying clusters from the scatter plot of moments. The dark image background is subtracted form the fluorescence image and the intensity is corrected for the field of light. Before intensity evaluation, the local background caused by adsorption of the label on the microplate bottom is subtracted from the label intensity image. The background image is obtained from morphological image opening applied to the intensity image. A disk-shaped kernel of 30 pixel diameter is used to remove beads in the background image. Finally, the fluorescence intensity of each bead is evaluated in the fluorescence image by computing the mean intensity of all pixels within 3 pixel distance from the bead centre that was localised in the brightfield image. Signals are normalised to an integration time of 100 ms if not stated otherwise.

Results and discussion

Choice of labels

Labels are chosen to be spectrally compatible with the encoding concept for microsphere sedimentation array that relies on phosphorescent Ruthenium-Metal-Ligand-Complexes (Ru-MLCs) in combination with other fluorescent dyes [5, 6]. Ideally, the fluorescence of the DNA label should be detectable without background from the encoding dyes. This criterion is fulfilled by fluorescent labels with either emission below 530 nm or excitation

Table 1 Properties of fluorescent labels characterised in magnetic bead sedimentation arrays

Name	Label type	λ_{ex} [nm][a]	λ_{em} [nm][a]	ε[b] [L/(mol*cm)][a]	Molecular weight or size[a]	Filter set[c]	CCD q.e[d] [%][a]
SYBRgreen I	Nucleic acid stain	497	520	N/A	N/A	475/515	38
Fluorescein	Direct	491	514	92,300	322 g/mol	475/515	39
Alexa488	Direct	495	519	71,000	643 g/mol	475/515	38
Cy5	Direct	650	667	250,000	975 g/mol	640/690	14
PBXL-3	Sandwich	614	662	100,000,000	80 nm (long axis)	640/690	15
R-phycoerythrin	Sandwich	480, 546, 565	578	1,960,000	240,000 g/mol	535/515	25
Allophycocyanin	Sandwich	650	660	700,000	104,000 g/mol	640/690	14
PD-Pt	Sandwich	405, 505	650	N/A	40 nm	405/610	15
Fluospheres505	Sandwich	505	515	N/A	43 nm	475/515	39
QD525	Sandwich	N/A	525	130,000 (at 488 nm)	5 nm	475/515	38
QD655	Sandwich	N/A	655	5,700,000 (at 405 nm)	10 nm	640/690	15
QD705	Sandwich	N/A	705	8,300,000 (at 405 nm)	11 nm	640/690	11

[a] Values are extracted and estimated from the product information of the manufacturers
[b] Extinction coefficient
[c] See Table 2
[d] Quantum efficiency of the CCD chip at the respective emission maximum of the label

Table 2 Filter combinations applied for the detection of various fluorescent labels

Filter set	Excitation filter	Dichromatic mirror	Barrier filter
475/515	Omega 475AF20	Omega 500DRLP	Chroma HQ515/30
640/690	Zeiss BP640/30	Zeiss FT660	Zeiss BP690/50
535/590	Zeiss BP510–560	Zeiss FT580	Zeiss LP590
405/610	Omega 405DF10	Omega 420DCLP	Schott RG610

above 610 nm. Note that in this study non-encoded beads were used. An overview of investigated fluorescent labels and their properties is given in Table 1. Although not spectrally compatible with the encoding dyes, R-phycoerythrin was included in the series as a reference because it is common label in multiplexed bead-based arrays [7]. PD-Pt nanoparticles were included because they exhibit decay-times time of approx. Seventy-five microseconds and therefore can potentially be used as labels in time-resolved fluorescence detection mode (not investigated here). Label performance was tested by hybridisation of target oligonucleotides to complementary capture probes covalently bound the surface of microspheres. Fluorescent labels were applied either covalently bound to target oligonucleotides or as streptavidin conjugates, which bind to biotin-labelled oligo-nucleotides subsequent to hybridisation. These different assay formats were chosen according to their frequency in reported suspension array studies.

Signal intensities

A series of fluorescent labels were investigated with the aim to reveal the most suitable for the application in microsphere sedimentation arrays. For a quantitative performance comparison DNA hybridisation assays on micro particles were performed under identical conditions according to the protocol described in the "Materials and methods" section.

The signal level is an important criterion but even more important is the comparison of signal and background. Therefore the signals of target and blank samples were recorded. Blank signals for sandwich-type labels and SYBR green are measured by challenging labels with non-hybridised capture probe beads. Direct labels are brought together with beads carrying a non-complementary sequence. A graphic representation of the signals and the blank signals and the signal-to-background ratio is given in Fig. 1.

Green emitting Quantum dots (QD525) excited at 475 and red emiting QD (QD655, QD705) excited at 650 nm show moderate emission, lower than organic fluorophores Alexa488 and Fluorescein. On the other hand, QD655 excited at 405 nm exhibit very intense signals. This can be explained with the increasing absorption coefficient of QDs towards UV-wavelengths. Hence, red emitting QDs have brighter emission when excited with 405 nm. However, this combination of excitation and emission is prone to spectral interference from encoding dyes (Ru-MLCs). Moreover, QDs exhibit a high blank signal and low signal-to-background ratio due to non-specific adsorption as illustrated in Fig. 2.

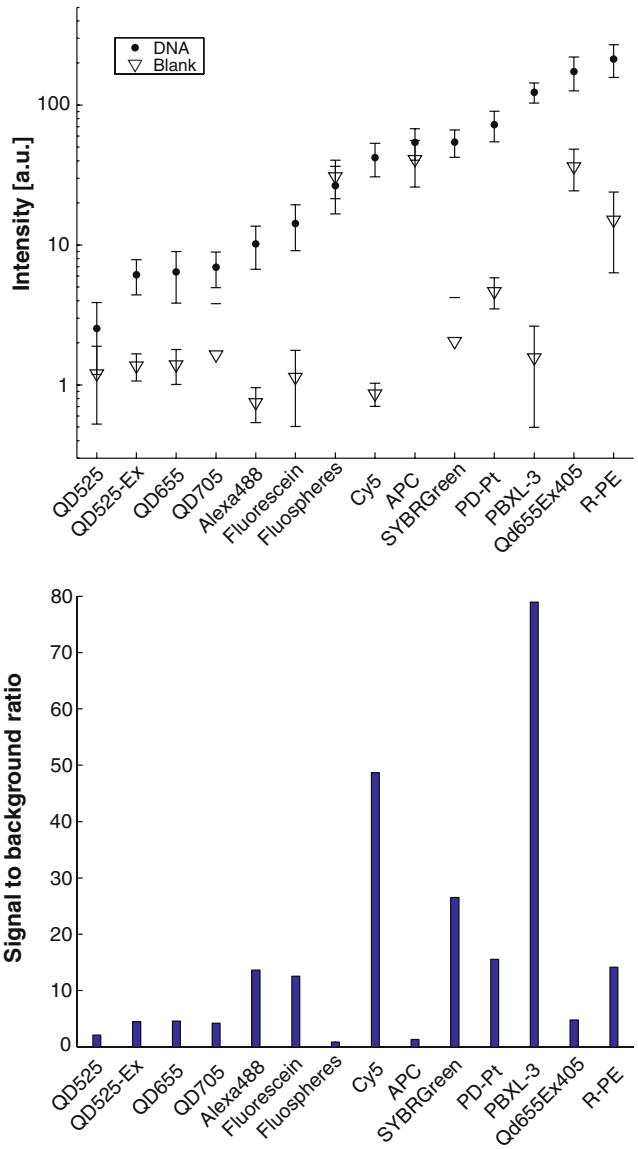

Fig. 1 Comparison of labels for oligonucleotide detection using microsphere arrays. The average of 30 microspheres in two images was evaluated for signal of hybridised complementary oligonucleotides and blank samples, respectively (*top*). The signal-to-background ratio gives an overview of label performance (*bottom*)

In contrast, blank signals of fluorescein, Alexa488 and Cy5 are identical to the background of imaging noise that remains after morphological image opening applied for background correction.

SYBRgreen yields high signal but high and non-uniform background (indicated by the extended error bar). The level of background can be partially explained by staining of single stranded capture oligonucleotides [14]. Although SYBRgreen is double strand specific, low but detectable fluorescence is emitted from single stranded oligonucleotides. Additional background signal arises from impurities from other external influences, e. g. buffers and microplates.

FluoSphere's yield high signal intensities, but blank and positive assay signals are hardly distinguishable due to non-specific binding and adsorption. The same observation was found for phycobiliprotein APC. PD-Pt-nanospheres were excited at 405 nm show good results with comparably high signal intensities. As described above this excitation wavelength interferes with the spectra of the encoding dyes.

The strongest fluorescence signal is detected for R-PE. This is explained by a high extinction coefficient of 1,960,000 L/(mol*cm) and a quantum yield 0.82. On the other hand, the background of R-PE is significantly high. This results in a comparably low signal-to-background ratio. Note that the application of this label in microsphere sedimentation assays is not possible, because R-PE is not spectrally compatible with the encoding dyes.

Table 3 Comparison of label performance for oligonucleotide detection in bead sedimentation arrays

Label	Signal [a.u.][a]	Blank [a.u.][a]	Signal/ background ratio	Photostability [% after 1 min]
SYBRgreen I	54.4	2.1	26.5	23
Fluorescein	14.3	1.1	12.5	32
Alexa488	10.2	0.7	13.6	71
Cy5	42.1	0.9	48.7	39
PBXL-3	123.6	1.6	79.0	81
R-phycoerythrin	54.1	41.0	1.3	25
Allophycocyanin	213.6	15.1	14.1	85
PD-Pt	72.4	4.7	15.5	72
FluoSpheres505	26.6	30.8	0.9	88
QD525	2.5	1.2	2.1	n.d.
QD655	6.4	1.4	4.6	108
QD705	6.9	1.6	4.2	n.d.

Photostability as % of the initial signal after 1 min of illumination with corresponding light source and filter set

Cy5, one of the most popular organic labels, shows a high fluorescence signal and low and uniform background that is comparable with the instrument noise.

The best overall performance was found for PBXL-3 with the highest signal-to-background ratio and third highest overall signal. The performance of all investigated labels is summarized in Table 3.

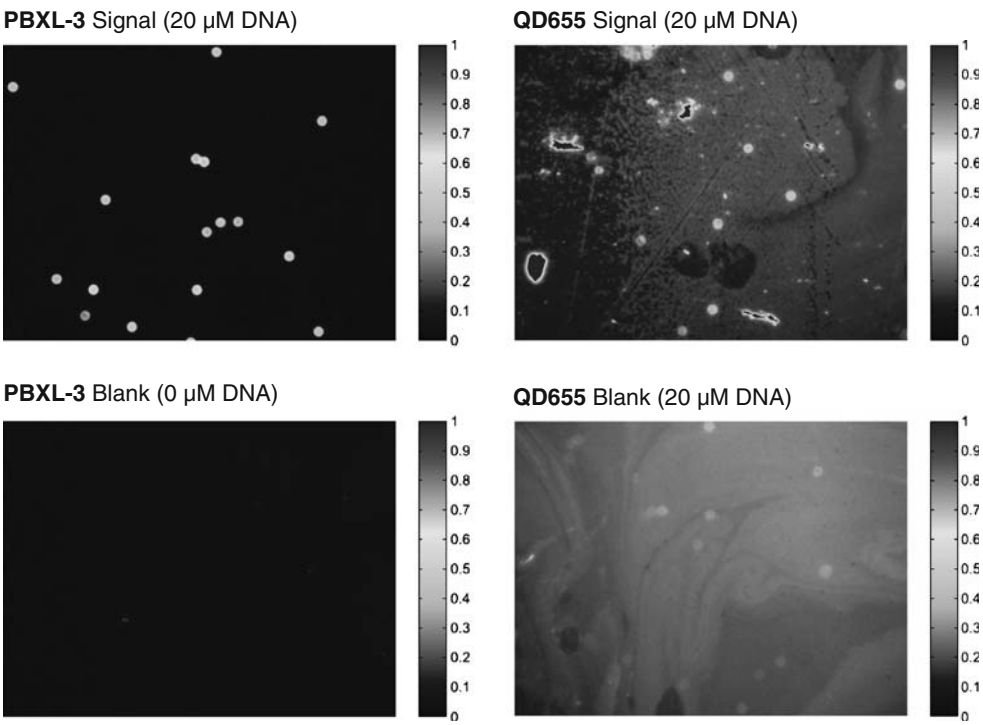

Fig. 2 Images of uncoded beads as carriers in DNA hybridisation assays. Field of view is 330×250 µm. PBXL-3-streptavidin conjugate used as label (*left*). QD655-streptavidin conjugate used as label (*right*). Positive signal of hybridised complementary oligonucleotide (*top*). Blank signal due to non-specific binding of the label (*bottom*)

Background signal from unspecific absorption

The suitability of a label depends also on the tendency to bind to the microplate surface or the carrier itself. The unspecific adsorption yields in background fluorescence and consequently impairs the signal-to-background ratio. The problem of unspecific adsorption is illustrated by two examples in Fig. 2. Characteristic images are shown when applying different sandwich-type labels. It is clearly visible, that PBXL-3 yields low blanks and good signal uniformity. QD have a tendency to form aggregates and to adsorb to the surface of the microplate and to microspheres. Similar behaviour was found for FluoSpheres and allophycocyanine. Non-specific adsorption of QDs was also reported to be an issue when applied as label in microplate assays and for in-vivo imaging [13, 15]. Even multiplexed assay systems using quantum dot encoded polymer beads as carriers rely on conventional labels like streptavidin-R-phycoerytrin-Cy5 [16] conjugates or Cascade blue [17] for detection. Up to now, no reports of QDs applied as fluorescent label in bead-based assays were found.

Photobleaching

Fluorescent labels with good photostability are desired for the purpose of the application in sedimentation arrays because of the necessary assay preparation and evaluating steps. The photobleaching behaviour is investigated applying the same assay procedures a described before. Microspheres with captured labelled oligonucleotide were illuminated over a period of 5 min in the fluorescence microscope using the appropriate filter sets. The results are graphically illustrated in Fig. 3 and the percentage from initial intensity after 1 min is listed in Table 3. R-PE, Fluorescein, SYBRgreen and Cy5 lose more than 60% of the initial fluorescence intensity within 1 min. Alexa488 and PD-Pt show moderate loss of approx. 30% in 1 min. Sufficient performance was found for FluoSpheres, APC and PBXL3 with a loss of 12%, 15% and 19%, respectively. An enhancement of the initial fluorescence signal was found for QD655. This behaviour has been reported and is attributed to electron and hole traps from impurities and at the surface and to photo-induced surface healing [18].

Quantification

Calibration experiments were carried out to characterize the performance of the detection system. The dynamic range for a quantitative detection of DNA was determined by a serial dilution of target oligonucleotides in the range from 10^{-10} to 10^{-8} mol/L. Alexa488-labelled oligonucleotides and streptavidin-PBXL-3 conjugate was used for the sandwich-type assays of biotin labelled oligonucleotides (Fig. 4). Two to

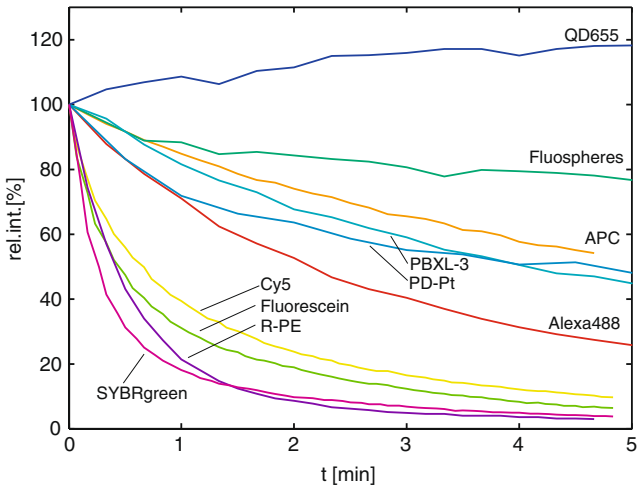

Fig. 3 Photobleaching of labels illuminated in a fluorescent microscope with the appropriate filter combinations. Intensities were normalised to 100% at t=0 s

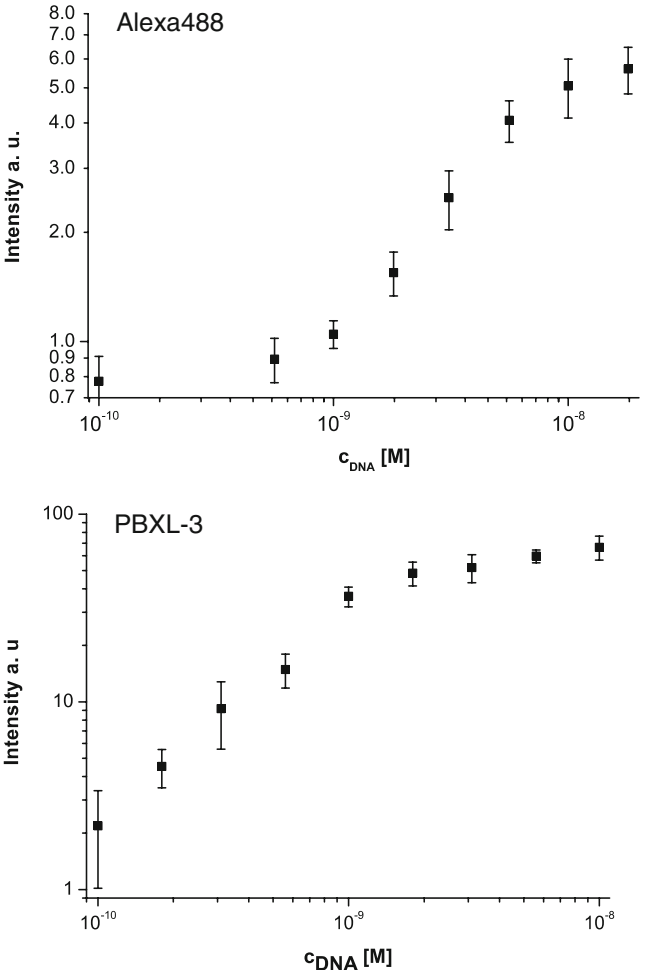

Fig. 4 Calibration curve for DNA hybridisation assays using Alexa488-labelled target (*top*) and PBXL-3 as reporter in sandwich assay format (*bottom*)

four images were evaluated for each concentration, displaying an average of 32 beads per concentration. Median value and inter quartile range (IQR) where used as statistical parameters, because they are more robust to outliers than mean value and standard deviation.

A dynamic range between 10^{-9} and 10^{-8} mol/L was determined for Alexa488 (Fig. 4 Top). A constant level of background noise is detected at lower concentrations. The signal converges to a constant level at concentrations higher than 10^{-8} mol/L. This effect can be explained by saturation of binding sites at the bead surface and was also reported for other bead-based assays [19]. Extension of the dynamic range towards lower concentrations could possibly be achieved by applying a CCD with lower electronic noise and longer integration times, and filter combinations with lower background fluorescence. However, background from the encoding fluorophores also has to be considered and is the limiting factor when applying encoded beads as assay carrier.

Alternatively, a label with higher signal intensity is expected to extend the dynamic range towards lower concentrations. Serial dilution of PBXL-3 reveals signal-concentration dependence from 10^{-10} to 10^{-9} mol/L as shown in Fig. 4 Bottom. The limit of detection was 2×10^{-10} mol/L. As expected, the lower detection limit is shifted to lower concentrations by one order of magnitude. Interestingly, the upper limit of the dynamic range is also shifted to lower concentrations by approximately the same interval. Taking into account the size of the labels, this finding can by attributed to sterical effects. Phycobilisome PBXL-3 with 80 nm largest diagonal is considerably more bulky than organic molecules like Alexa488 with a molar mass of 643 g/mol. Consequently, the loss of measuring capability at higher concentrations can be interpreted as complete surface occupancy by the PBXL-3 label.

These results are comparable with reported values for measurements with flow cytometers. Minimum detectable concentrations of 1 fmol in 60 μL hybridisation buffer, which equals 1.6×10^{-11} mol/L were obtained for a Luminex system when quantifying cDNA obtained by PCR [19]. The hybridisation signal was reported to be proportional to the target concentration from 1 to 100 fmol using streptavidin-R-phycoerythrin as fluorescent label. Spiro et al. found similar dynamic ranges from 1 to 100 fmol for the detection of PCR product, with a limit of detection of 1 fmol in 6 μl sample volume, equalling a concentration of 1.6×10^{-10} mol/L [20, 21]. The working concentration of analyte determination for the imaging system applied here and flow-cytometers are in comparable range, although based on intrinsically different instrumental approaches. Flow cytometers are equipped with lasers as excitation light source and sensitive photomultipliers for detection of fluorescence signal. On the other hand, because of the sequential read-out the exposure time is relatively short, typically in the microsecond domain. In contrast, the imaging system relies on less powerful excitation and detection equipment, namely an arc lamp and a CCD camera. This is compensated by extended exposure times of up to 1 s and parallel readout of a multitude of beads, permitted by the two-dimensional detector.

A difference is observed in dynamic ranges, which are reported to be 1.5 to 2 orders of magnitude in the flow cytometric systems, while 1 order of magnitude is achieved with the fluorescence microscope imaging system applied here.

Conclusions

Assay evaluation by the fluorescence microscope imaging system allows determination of oligonucleotides with detection limits down to 2×10^{-10} mol/L, depending on the fluorescent label. This is comparable with typical flow cytometric systems. A drawback is the reduced dynamic range of one order of magnitude. Non-specific binding turned out as the major obstacle for the application of some high performance labels like Fluospheres, allophycocyanin and quantum dots. Further improvement of the system sensitivity is anticipated if microscope objectives with higher numerical aperture are used.

PBXL-3 is the label of choice for detection of oligonucleotides at concentrations from 2×10^{-10} to 10^{-9} mol/L because of its low unspecific binding, high signals intensity and signal-to-background ratio. In concentration ranges from 10^{-9} to 10^{-8} mol/L, directly labelled oligonucleotides are preferred because no additional incubation and washing steps are required. Herein, Cy5 exhibits good signal-to-background ratio as red fluorescent label, but suffers from a poor photo-stability. For the green detection channel Alexa488 is superior to Fluorescein because of it's significantly better photostability.

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