

Photochemically amplified detection of molecular recognition events: an ultra-sensitive fluorescence turn-off binding assay†

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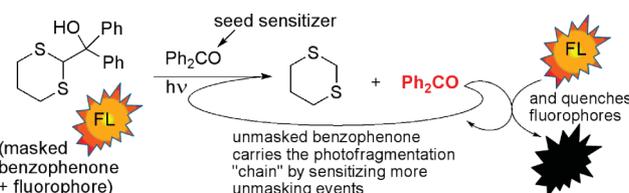
Amplified fluorescence quenching methodology based on massive autocatalytic photo-unmasking of a dual function sensitizer–quencher is developed and adopted for photoassisted ultra-sensitive detection of molecular recognition events. The resulting binding assay, based on a molecular recognition-triggered photo-amplified cascade with concomitant decrease of fluorescence is validated with the biotin–avidin pair, achieving attomolar detection.

Fluorescence-based screening for binding events on spatially addressable chips has revolutionized biomedical research. Yet, with all the remarkable advances in hardware and the surface density of chips, it is premature to expect mass produced scanners capable of single molecule detection levels any time soon. For any given hardware detection limit the question then remains: can one chemically pre-amplify the signal to a detectable level (or alternatively turn-off the fluorescence from an easily detectable level)? PCR is a powerful method for such pre-amplification, but it is limited to genetic material. In a more general sense, beyond classical enzymatic catalysis,¹ amplification can be achieved with polymerization;² yet the readout is less sensitive than fluorescence. There are several powerful non-PCR methods, for example: Mirkin's biobarcode assays³ and PCR-like cascade reactions,⁴ Abbott's liquid crystal reorientation,⁵ or a sensitive fluorescence turn-off approach based on "superquenching" of conjugated polymeric chromophores.⁶

In this Communication we report a fundamentally different methodology for amplified fluorescence quenching based on massive autocatalytic photo-unmasking of a quencher, and its implementation for ultra-sensitive detection of molecular recognition events. We have validated this photoamplified fluorescence turn-off concept with the biotin–avidin binding pair, which allows the full range of physiologically relevant K_D values to be probed. Utilizing a mass produced consumer CCD camera (no chip cooling) we achieved a reproducible detection of 50 attomoles of avidin.

We previously reported photoassisted detection of molecular recognition events in solution based on a spatial proximity test

involving externally sensitized fragmentation in dithiane–ketone adducts.⁷ Next a system was designed where a seed sensitizer triggers a chain of fragmentation events.^{8,9} As fluorescence detection is one of the most sensitive techniques, we have now developed a methodology of conditional, *i.e.* molecular recognition-triggered, photoamplified release with *concomitant fluorescence quenching* (Scheme 1) based on benzophenone (BP) acting as *both* the amplification chain carrier *and* the quencher for the reporter fluorophore. Depending on the fluorophore, the emission turns off either because of photophysical quenching by the amplified BP, or an actual photochemical reaction with triplet excited BP irreversibly bleaching the fluorophore.



Scheme 1 General concept for the massive amplified unmasking of benzophenone resulting in fluorescence quenching (see text).

As shown in Scheme 1, masked BP is mixed with the reporter fluorophore, which is chosen to have negligible absorption around 365 nm, *i.e.* the wavelength reserved for photoamplification.

Irradiation at 365 nm does not exert any effect until a seed sensitizer is brought to the pool of masked sensitizers, for example, by a molecular recognition event. At this point externally sensitized photofragmentation of masked benzophenone is commenced, unmasking more BP, which in turn induces more fragmentation, thus carrying the amplification chain. The unmasked/amplified BP turns the emission of the present fluorophore off, signifying a positive "hit". Unlike radical chain polymerization, the propagation of this photoamplified chain can be halted by interrupting the UV source. This feature is critically important to prevent "overdevelopment" of the image and avoid false positives.

First we examined fluorophores with high-lying singlet states, for example, polyphenyls, which are readily quenched with amplified benzophenone *via* a bimolecular collisional energy transfer mechanism with a very high Stern–Volmer constant. A concentration series with quaterphenyl as a fluorophore is shown in Fig. 1. The fluorophore-masked BP formulation is seeded with progressively diluted BP, increasing the autocatalytic delay time.

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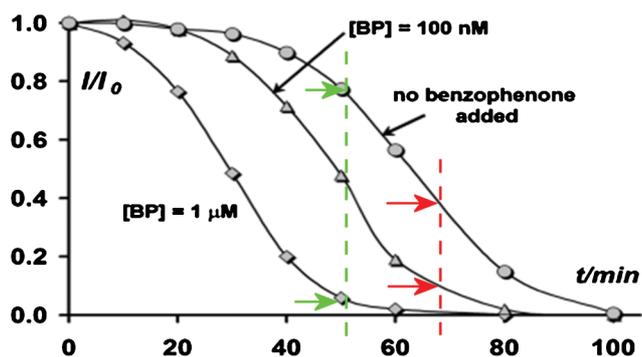


Fig. 1 Quenching of quaterphenyl as a result of benzophenone (BP) amplification (y -axis is the normalized emission at 360 nm).

At a certain dilution, under 10 nM, the resulting fluorescence intensity is no longer different from the “no benzophenone added” curve. This is the intrinsic detection limit, which is defined by two factors, (i) the extent of non-sensitized fragmentation of the masked sensitizer, *i.e.* its spontaneous monomolecular fragmentation under direct absorption of light, generating enough seed BP to mistrigger amplification (a false positive) and/or (ii) the residual levels of free BP in the adduct, initiating amplification chain even in the absence of a molecular recognition event. The latter is rectified by a thorough purification of the masked sensitizer from residual BP, while the former can potentially be improved by further conditioning of the UV light used in the amplification: a narrow UV source which selectively excites the $n \rightarrow \pi^*$ of benzophenone, while avoiding direct shorter wavelength excitation of the deconjugated phenyl moieties in the masked sensitizer.

The emission differences between the test samples and the control increase and then decrease in time, as all masked sensitizer is eventually released. As the system has a built-in off switch, where discontinuing irradiation stops amplification, false positives are prevented by optimizing the time it takes to obtain the maximum fluorescence difference. For example, the 1 μM sample reaches a 1 : 15 emission ratio, as the vertical green line shows, whereas for a smaller concentration of 100 nM it is about 1 : 4 (red line).

Fluorophores optimized for visible light microscopy are more suitable for ready detection with mass-produced CCDs than those emitting in the UV. Inherently, they have low-lying singlet states, which are not quenched as effectively by benzophenone. However, the excitation and intersystem crossing of the amplified BP produces reactive triplet species, which *can chemically destroy the fluorophore achieving the same turn-off effect*. We tested several bright fluorophores and chose coumarin-6 (C6, structure is shown in Fig. 4), which in the presence of triplet benzophenone undergoes a cascade de-ethylation reaction with concomitant loss of emission.¹⁰ This photochemical reaction partially depletes the amplified benzophenone, but this depletion is negligible and does not affect the amplification chain because the masked sensitizer pool, 30–40 mM, is more than three orders of magnitude greater than the total amount of fluorophore, present at 10–20 μM (see SI for an NMR-monitoring experiment).

The current state-of-the-art in microarray screening for binding takes advantage of (i) ligands modified chemically to immobilize on spatially addressable chips and (ii) a biological target which is chemically outfitted with a fluorophore. Binding events sequester

a few emitting copies of the biological target to a certain spot on the surface of the chip. The success of such an assay depends on detecting a very limited number fluorophores, *i.e.* requires high end X-Y fluorescence scanners.

We contend that in this general scheme the Z-direction is underutilized and that without sacrificing the surface density of an array one can utilize the *depth* of the screening chip (for example, by using a now ubiquitous microcapillary array chip¹¹), its pores filled with enough fluorophore for fast detection/imaging with inexpensive CCDs. Fig. 2 shows that for a typical focus depth of an unsophisticated two lens imager (see SI for details) fluorescence detection does benefit from the additional fluorophore stored in the Z-direction: 0.86 mm ID capillaries were loaded with increasing volumes of 10^{-5} M coumarin-6 in dichloromethane, where 1 $\mu\text{L} \approx 1$ mm in depth. A clearly discernible increase in average pixel intensity was obtained by increasing the depth of the fluorescing solution from 1 to 4–5 mm, at which point the intensity levels off (corresponding to an approximate 7 : 1 ratio of the depth to ID). For a microcapillary array chip with 200 μm pores this translates into a useful depth of 1.4 mm and the corresponding pore volume of >30 nL, containing near picomolar amounts of fluorophore which is easily detectable with a simple imager.

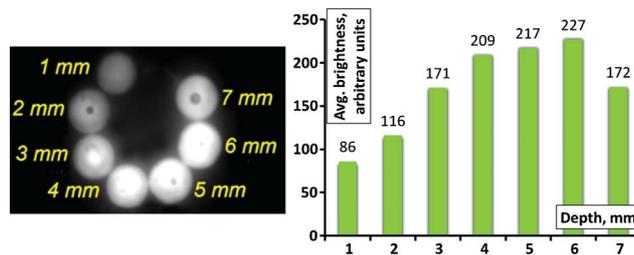


Fig. 2 Effect of path length on fluorescence intensity as imaged by the CCD camera. Each capillary shows an increase in average pixel intensity (graph on the left) which correlates with an increase in the depth of the 10^{-5} M coumarin-6 solution.

Fig. 3 outlines the general concept for amplified detection in compartmentalized small volumes: (A) Microwells are uniformly loaded with a fluorophore and masked sensitizer in organic solvent or organogel. This formulation is generic and universally applicable to any type of assayed molecules. The ligands, tethered to an amphiphile, are printed on the surface of wells and therefore displayed at the organic–aqueous interface. (B) A sensitizer-tethered target protein is added in buffer and incubated to allow for binding. A binding event brings the tethered sensitizer to the solvent interface at which point irradiation commences. (C) Initially the inserted sensitizer unmasks a few copies of BP in its immediate vicinity. These benzophenones are free to diffuse

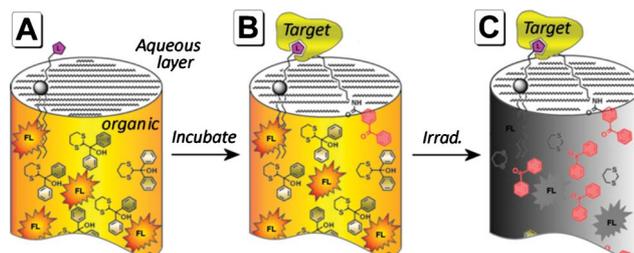


Fig. 3 Photoamplified detection of a binding event (see text).

throughout the entire volume of the well, releasing and amplifying BP en masse, which lead to complete quenching/bleaching of the fluorophore. As a result, the whole well/pixel goes dark indicating a “positive hit”.

We validated this concept using the biotin–avidin pair which remains bound in a broad dynamic range of concentrations, and thus allows for probing of the method’s intrinsic detection limit. Commercial biotin-capped dipalmitoyl phosphoethanolamine or its chain length variants, synthesized by tethering biotin to other phosphatidyl ethanolamines, were used in this study. Avidin was outfitted with xanthone as the seed sensitizer (for experimental details refer to SI).

Biotin–avidin binding failed to initiate the amplification cascade when the initiator’s tether was too short (C_{11}) due to insufficient lipid layer penetration. However, doubling the tether’s length to ~3 nm long, allowed for an efficient initiation of the photoamplification chain.

Fig. 4 shows a “bulk” series where large 700 μL fluorescence cells were loaded with 300 μL solution of 30 mM dithiane-masked BP (structure in Scheme 1) and 10 μM fluorophore C6. At the organic–aqueous interface either biotinylated lipid or phosphatidylcholine (POPS, control) was used. The avidin–xanthone conjugate, or blank PBS buffer as a control, was added in phosphate buffered saline (PBS).

Upon irradiation, a 10-fold decrease of emission was detected in the cell with both biotin and avidin present (Fig. 4A). Emission in the cells lacking avidin (Fig. 4C,D) decreased negligibly. Of the controls, fluorescence in the cell containing avidin–xanthone, but not biotin, was reduced by approximately 35% (Fig. 4B), indicating that, at 10 μM , a small amount of avidin is partially recruited to the lipid interface by non-specific binding. However, this is still six fold brighter than in (A), thus allowing for easy identification of a “positive hit”.

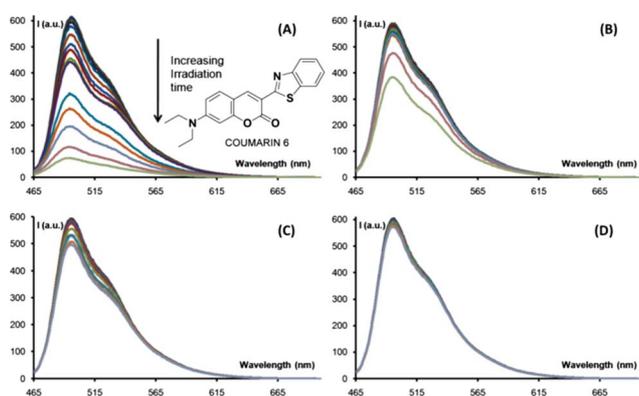


Fig. 4 A–D contain C6 (10^{-5} M) and masked sensitizer (30 mM) in 1,4-dichlorobutane. **A**, **C** biotin capped lipid (9.5×10^{-5} M) is added; **B**, **D** POPS (9.5×10^{-5} M) is added. **A**, **B** were incubated with 0.01 M PBS pH 7.5 containing the avidin–xanthone conjugate (10^{-5} M) while **C**, **D** were incubated with 0.01 M PBS pH 7.5 as control, before irradiation. **A** is the only sample containing both biotin and avidin.

We then emulated the micro-well environment with capillaries, either 0.86 mm i.d. sealed from one end, or 0.4 mm open from both ends (the latter loaded by capillary forces). Fig. 5A shows images of the dilution series, where a 3 μL droplet of masked BP and C6 in dichlorobutane was incubated with either a control of 5 mL PBS

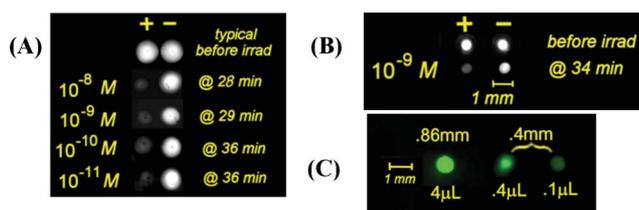


Fig. 5 (A) Bottom view pair wise comparison of the 0.86 mm i.d. capillaries in the dilution series with (+) and without (–) avidin–xanthone conjugates. The best contrast is achieved at around 30 min of irradiation. (B) a typical fluorescence turn-off outcome in an open ended 0.4 mm i.d. capillary at 1 nM avidin–xanthone. Images are acquired with a 0.5 s exposure of a mass produced 1/4” CCD. (C) Images of capillaries with 4, 0.4, and 0.1 μL of 10^{-5} M C6 acquired with a cell phone camera.

lacking the avidin–xanthone conjugate “(–)” or a 5 μL aqueous solution of the progressively dilute avidin–xanthone conjugate, “(+)”. The capillaries were then subjected to gentle irradiation with 365 nm 250 mW Nichia UV LED. All the necessary control experiments were run in parallel (see SI).

As follows from Fig. 5A the avidin containing spots and spots lacking avidin exhibited the same emission level before photoassisted amplification (top). Within approximately 30 min of irradiation a reproducible fluorescence turn-off effect was achieved in the capillaries containing avidin, while the control capillaries lacking avidin remained brightly lit, offering an excellent contrast ratio for detecting the “positive hits”. Photoamplification of biotin–avidin binding events was thus reproducibly observed down to 10 pM avidin which corresponds to a total of 50 attomoles – a remarkable achievement for a non-cooled CCD. This is comparable to other binding assays, including Mirkin’s bio-barcode assay which can detect ~10 attomoles of analyte,¹² and Bowman’s visible-light-polymerization which can detect 0.4 attomoles of biotin with the help of a research grade microscope.¹³

Although several successful runs were recorded for 1 pM avidin, the reproducibility below 10 pM was poor. As biotin is still expected to be mostly bound at this concentration, we interpret this as the intrinsic detection limit for our photoamplified turn-off assay. Remarkably, the observed detection limit of 10 pM was considerably better than the amplification of untethered BP in a bulk solution (Fig. 1). We rationalize it in terms of a local pre-concentration effect due to recruitment of the avidin–sensitizer conjugate to the biotinylated lipid interface.

The spatial resolution of mass-produced CCDs is fully adequate for imaging high density arrays. However, their sensitivity of detection leaves much to be desired, *i.e.* they simply cannot image a few fluorophore molecules sequestered to a 2D spot on a surface of a microarray chip. Utilizing the third – depth – dimension we can image as little as 100 nanolitres of coumarin-6 with such a ubiquitous imaging device as a cell phone camera (see Fig. 5C). For a 2–3 mm thick microcapillary array chip this translates into a surface density of pores exceeding 10^4 per square inch.

As long as one can image the initial level of emission, the photoamplified fluorescence turn-off assay can be successfully carried out. This offers ultra-sensitive yes/no bioanalytical capabilities which can be developed for situations when access to state-of-the-art technology is limited.

For the high-end instrumentation, with sub-picolitre volumes of fluorophores and scientific grade cooled CCD cameras our

photoassisted pre-amplification methodology could potentially approach a single molecule detection limit. Again, to reiterate, the uniform loading of the microwells with a fluorophore and masked sensitizer makes this formulation generic and universally applicable to any type of assayed molecules.

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