Published in final edited form as:

Anal Biochem. 2017 August 15; 531: 20–36. doi:10.1016/j.ab.2017.05.020.

Radiative Decay Engineering 8: Coupled Emission Microscopy for Lens-Free High-Throughput Fluorescence Detection

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Abstract

Fluorescence spectroscopy and imaging are now used throughout the biosciences. Fluorescence microscopes, spectrofluorometers, microwell plate readers and microarray imagers all use multiple optical components to collect, redirect and focus the emission onto single point or array imaging detectors. For almost all biological samples, except those with regular nanoscale features, emission occurs in all directions. With the exception of complex microscope objectives with large collection angles (NA > 0.5), all these instruments collect only a small fraction of the total emission. Because of the increasing knowledge base on fluorophores within near-field (< 200 nm) distances from plasmonic and photonic structures we can anticipate the development of compact devices in which the sample to be detected is located directly on solid state detectors such as CCDs or CMOS cameras. Near-field interactions of fluorophores with metallic or dielectric multi-layer structures (MLSs) can capture a large fraction of the total emission. Depending on the composition and dimensions of the MLSs, the spatial distribution of the sample emission results in distinct optical patterns on the detector surface. With either plain glass slides or MLSs the most commonly used front focal plane (FFP) images reveal the x-y spatial distribution of emission from the sample. Another approach, which is often used with two or three-dimensional nanostructures, is back focal plane (BFP) imaging. The BFP images reveal the angular distribution of the emission. The FFP and BFP images occur at certain distances from the sample which is determined by the details of the optical components. Obtaining these images requires multiple optical components and distances which are too large for the compact devices. For devices described in this paper, the images will be detected at a fixed distance between the sample and some arbitrary distance below

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Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org>

the MLS which is determined by the geometry and thicknesses of the components. We refer to measurements at these locations as out-of-focal plane (OFP) imaging. Herein we describe a method to measure the optical fields at micron and multi-micron distances below the MLS, which will represent the images seen by an optically coupled array detector. The possibility of sub-surface optical images is illustrated using five different multi-layer structures. This is accomplished using an optical configuration which allows measurement at a front focal plane (FFP), back focal plane (BFP) or any OFP locations. Our OFP imaging method provides a link between the FFP images which reveals the surface distribution of fluorophores with the BFP images that reveal the angular distribution of emission. This linkage can be useful when examining structures which have nanoscale features due to fluorescence or leakage radiation from nanostructures.

Abstract

Keywords

Radiative Decay Engineering; Coupled-Emission Microscopy; Focal Plane Imaging; Back Focal Plane Imaging; Out-Of-Plane imaging; Coupled Emission; Metal Dielectric Waveguides; One-Dimensional Photonic Crystals; Tamm States; Tamm State-Coupled Emission

Introduction

Starting in the 1960's, fluorescence spectroscopy has been increasingly used for biochemical research [1–3]. The initial applications of fluorescence spectroscopy focused on biophysical studies of proteins, membranes, nucleic acids and their distributions within cells. The information content of fluorescence spectroscopy has been greatly increased by the availability of time-resolved decays and the software to analyze these data [4–5]. The time-resolved data contain information about the structure and dynamics of macromolecules, including measurements of site-to-site diffusive motions within a single macromolecule or a complex of macromolecules [6–7]. During this time there has been a parallel growth in the use of fluorescence microscopy in cell biology and imaging [8–10]. Cell imaging is now performed using a variety of techniques to improve the spatial resolution such as confocal optics, multi-photon excitation, super-resolution by STED, STORM or PALM technologies [11–13] and by using light-sheet microscopy [14–15]. Fluorescence detection is also used in biomedical applications such as medical diagnostics, drug discovery, nucleic acid sequencing and genetic testing [16–17]. This expanding use of fluorescence is likely to include point-of-care and self-testing devices. Initially, most fluorescence measurements were performed using homogeneous solutions with multi-ml volumes. At present many

applications rely on fluorophores localized near glass surfaces by chemical linkage or by capture proteins, which are then modified to bind target molecules by biomolecule association reactions [18–19]. These binding events are often measured using microwell plates, spotted arrays or sample holders designed for specific analytes. The feature (well) sizes on microwell plates are typically near 5 mm, but for high density microwell plates and spotted arrays for genomics or proteomics the feature sizes can be as small as 25 microns (μm) [20–25]. In principle, the emission from microwell plates or spotted arrays could be imaged using wide-field optics and low magnification imaging. However, the fluorescence collection efficiency is greatly decreased with low numerical aperture (NA) lenses, and it is not practical to construct lenses which are large enough to efficiently collect the emission from large areas. The use of wide-field optics often yields low contrast images because the optical system collects emission from all depths in the samples including those which are outside the focal plane. For these reasons many microwell plate and printed array imagers use point-by-point (PbP) scanning, high NA objectives, and confocal optics to select emission from only the focal plane [26]. For similar reasons, laser scanning and confocal optics have become routine in fluorescence microscopy and cell imaging [27].

The use of higher magnification and larger NA optics results in a very limited field-of-view (FoV). For typical microscopes with 10, 40 and 100X objectives, with a 10X eyepiece, the FoV are near 2 mm, 450 μm and 180 μm , respectively. This limited FoV is the reason that most microwell imagers and microscopes use point-by-point scanning and complex mechanical and/or optical systems to image large areas. A wider FoV with a simple optical configuration would be valuable for many applications in addition to array imaging. For example, a pathologist needs to view slides at high magnification for close examination of boundary regions in a biopsy specimen. It is also necessary to examine the entire slide at lower magnification to confirm the diagnosis is representative of the entire slide. The need for both large area and high magnification has resulted in ongoing efforts to find practical approaches to whole slide imaging and archiving of high resolution images [28].

Why are complex optics required for fluorescence detection and imaging?

The sensitivity of fluorescence detection is limited by the photon count rates which can be obtained from fluorophores and by background emission from the samples. The fluorophores brightness is limited by a number of factors. Fluorescence occurs in all directions (omnidirectional) and in a typical spectrofluorometer only a small fraction of the emission can be detected. For instance, based on geometry, a 1 inch diameter lens located 3 inches from the sample can only collect 0.68% of the emission. The amount of collected light is reduced further by the factor $(n_o/n_s)^2$ where n_s and n_o are the refractive indices of the air and sample holder respectively [29–30]. For a water-to-glass-to-air interface this factor is close to 0.563 and about half of the emission leaving the sample is lost due to this effect of refraction. This loss can be avoided using an immersion objective. Additionally emission loss occurs with each optical element due to reflection, scattering or absorption.

At a more fundamental level the fluorescence intensity, relative to background from scattering or auto-fluorescence, is determined by the optical cross-sections for absorption. The small absorption cross-sections of fluorophores result in limitations due to unavoidable

background from the samples. Consider a single fluorophore in an excitation beam. The optical cross-section for absorption is about equal to the physical cross-sections or smaller, about 1 nm^2 [30–31]. According to quantum electrodynamics, every photon in a coherent light beam has the diameter of the beam. If the beam is not coherent the photon cross-section can be slightly smaller, but it cannot be smaller than the square of the wavelength. The probability of absorption is small because it equals the ratio of the fluorophore absorption cross-section to the much larger cross-section of the photon. The not-absorbed photons impinge on the water, biomolecules, impurities which can contribute unwanted background signal. Numerous approaches have been used to compensate for the limited brightness of fluorophores, including conjugation of multiple fluorophores to a single protein or dendrimer [32–33], the use of phycobiliproteins which have multiple chromophores [34], the development of semi-conductor quantum dots with high extinction coefficients [35] and the use of inorganic (silica) or polymer beads which contain hundreds of fluorophores [36]. In the present report we describe an approach which greatly increases the emission collection efficiency, possibly to an extent larger than using high NA immersion objectives. Our approach reduces background from the bulk solution by selective detection of surface-bound fluorophores because only fluorophores close to our structures can result in coupled emission. Moreover, our approach allows this collection efficiency and background suppression to be obtained over the large areas of CCD or CMOS cameras, without the use of additional optical components, lenses or free-space optics.

This laboratory [37–38] and others [39–41] have been applying the emerging knowledge in plasmonics and photonics to increase the brightness of probes for imaging and sensing, and to reduce contributions from unwanted background signals. In the case of nanoparticles these interactions have resulted in ultrabright probes [42–43], improved single molecule detection [44] and rejection of emission from the bulk sample for surface-bound assays [45]. Important results have been obtained with smooth continuous metallic or dielectric surfaces. In these structures the emission from fluorophores near the surfaces can be collected with high efficiency. Additionally, the multi-layer structure (MLS) can convert the usual isotropic emission distribution into well-defined angular distributions [46–49], which provides new opportunities for compact devices.

Our experience with metallic and dielectric nanostructures suggested an approach to bypass the complexity of current fluorescence technology (Figure 1). Our concept is to place multi-layer structures directly on the photosensitive surface of imaging CCD or CMOS devices, and/or to optically couple the MLS to the detector. The CMOS imaging detectors (CIDs) are rapidly replacing CCDs in many applications. The sensing surface in Figure 1 is shown to be spatially separated from the CID to illustrate the effects of a multi-layer surface (MLS) coating on the emission. In practice the MLS will be in direct contact with the CID. In this illustration we used a metal-dielectric waveguide (MDW). Since the refractive index of silicon is near 3.9 at visible wavelengths, and the refractive index of glasses are typically near 1.5, we do not expect total internal reflection before the detector. The illuminated fluorescent nanobeads (NBs) represent point sources of emission. Because of near-field coupling each NB creates a cone of emission which can be recorded by the CID. Many factors affect the coupled emission angles, diameters of the rings and the number of rings from each NB. These factors include the materials used in the layers, their thicknesses,

number of layers, and the solution above the structures. The development of devices similar to Figure 1 will require knowledge of the optical fields created by the sample or NBs which occur directly under the structure, and how these fields are affected upon propagation from glass or metal into the CID. In completed devices, which contain the combined MLS-CID components it may not be practical to measure these fields. Additionally, there will be a fixed distance between the sample and CID which is unlikely to provide FFP or BFP images. Hence a method is needed to correctly determine the subsurface optical fields under plasmonic and/or photonic structures.

In the present paper we describe a method to measure the out-of-focal plane (OFP) images which will appear at micron-scale distances below the top sensing surface. This method does not take into account near-field interactions between the MLS and CID, and may therefore not reveal the correct fields at distances below experimental wavelengths. To demonstrate the generality of this approach we use several multi-layer structures shown in Figures 2 and 3. Measurements of OFP images facilitate the design and construction of devices for coupled-emission microscopy (CEM) by providing direct measurements of the optical fields below any multi-layered structure. Here we use structures which do not contain any nanoscale features in the x-y plane. The use of our simple multi-layer structures, which do not require top down nanofabrication, allows low-cost fabrication of structures with large area of up to the size of slides or microwell plates. Combining these structures with rapidly evolving CMOS imaging technology will result in new classes of coupled emission devices for fluorescence detection in chemical analysis or medical testing.

This paper begins with a detailed explanation of BFP imaging, which is necessary to understand the OFP images and the potential of coupled-emission microscopy (CEM) for high-throughput fluorescence detection and compact medical diagnostic devices. The linkage between FFP, OFP and BFP images is complex because fluorescence on glass surfaces occurs in all directions, while the emission from MLS often occurs at specific directions through the MLS. An additional complication is the use of finite and infinity focused objectives in different microscopes. These different optical conditions and effects on the images are explained prior to presentation of the OFP images.

Materials and Methods

The multi-layer structures (MLSs) used in this report are shown in Figures 2 and 3. The individual layers are always thinner than the experimental wavelengths, and consisted of either silica (SiO_2), silicon nitride (Si_3N_4) or silver (Ag), with thicknesses ranging from 50 to 300 nm. The detailed dimensions and the optical constants are provided in the supplemental materials (Figure S1). Depending on the desired structures the layers were sputtered onto either glass microscope slides or cover slips. For measurements of coupled emission the MLS were then spin coated with solution of either polyvinyl alcohol (PVA) or polymethylmethacrylate (PMMA) which contained either rhodamine B (RhB) or Nile Red (NR) as described previously [48–49]. These films covered the entire FoV of the objectives. Focused laser excitation of these films provided point light sources. In some cases, to illustrate the difference between FFP and BFP imaging, the excitation beam was expanded to cover the entire FoV of the objective. To obtain multiple point sources of emission the

MLSSs were dropped or spin-coated with suspensions of 2 μm diameter fluorescent nanobeads (NBs) which were labeled with NR. Initially the angle-dependent emission intensities of the structures in Figures 2 and 3 were measured by illuminating from the top which is called the reverse Kretschmann (RK) side or configuration (Figure 4). From this direction the incident light cannot create surface plasmons, and any emission is due to near-field fluorophore-metal coupling [50–52]. Emission is measured from the bottom of the sample, which is called the Kretschmann (KR) side or configuration. For the macroscopic measurements using continuous fluorescent films, the angle-dependent coupled emission was collected using an optic fiber (1 mm in diameter) positioned 2 cm from the illuminated spot. A long pass filter was used to remove scattered light at 532 nm. A hemispherical prism was used for emission coupling so that the measured angle was the actual angle of the coupled emission inside the structure and there was no need for corrections due to refraction and the glass-air interface. For all figures the z-axis is along the optical axis, and the x-y plane is perpendicular to the z-axis and aligned with the FFP and BFP of the optics. The angles (θ) are measured from $\theta = 0$ starting at the axis perpendicular to the bottom of the structure (Figure 4). These measurements provide the angular emission distributions shown on the right sides of Figures 2 and 3. For some structures emission can occur at more than one angle as seen in the bottom row of Figure 2. The emission cones can have different polarizations (Figure 2 bottom row). S-polarization refers to the electric field perpendicular to the incident plane (or emission plane). This plane is defined by the propagation direction and a vector perpendicular to the structure. In our system, S-polarization refers to the electric field parallel to the MLS surface. P-polarization refers to the incident or emission field parallel to the incident plane, and/or to that portion of the light which is perpendicular to the MLS surface. This is true for both the incident light and light coupled through the MLS. Another approach to observing the coupled emission is to image the cone of emission with a CID. The optical configuration for imaging the entire cones is more complex and will be described in the Results section. Unless stated otherwise the focal plane images were obtained using an Olympus Apo N 60X/1.49NA oil immersion or an Olympus UApo N 100X/1.49NA oil immersion objective.

Results

Angle-dependent emission from MLSSs

To test the validity of our measurements of sub-surface optical fields and CEM we selected five structures and measured the angular emission distributions using the optical configuration shown in Figure 4. The first structure (in Figure 2) is a plain glass cover slip to serve as a control sample. When a glass surface is coated with a fluorophore and illuminated with a narrow beam the emission is distributed over a wide range of angles around the z-axis (Figure 2, top right panel). These angular plots of emission intensity can be confusing so these distributions on a linear x-axis angle scale are shown in Figure S2. If a fluorophore or point dipole source is located near a glass surface about 70% of the emission is coupled into the glass due to its higher refractive index, 1.52 as compared to 1.0 for air [53–54]. The emission is highest near the critical angle (ϕ_c) of 41. For a non-immersion microscope objective only the light at angles below ϕ_c can escape the glass and be observed. Water or oil-immersion objectives allow light above the glass-air ϕ_c to escape the glass and be

detected. The glass prism below the structure in Figure 4 is used to detect light emerging from the structure above φ_c .

The second structure is a glass slide coated with an about 45 nm thick Ag film, which we call a surface plasmon (SP) structure (Figure 2, middle). Because of the optical properties of the silver-air interface, RK incident light cannot create surface plasmons and is reflected. However, the incident light can excite fluorophores on the surface. Excited state fluorophores within about 50 nm of the Ag surface create surface plasmons [52]. This can occur because the real part of the wavevector projection onto the surface of the metal film can exceed the magnitude of the free electromagnetic wave in air. These dipole-induced surface plasmons can propagate through the Ag surface and radiate into a high refractive index prism with $n = 1.46$ for quartz or 1.52 for BK7 glass. This cone of emission is called surface plasmon-coupled emission (SPCE) [48–49]. The coupled emission angles depend on several factors including the thickness of the Ag film and polymer layer. The emission occurs over a relatively small range of angles. The surface plasmons and SPCE are both 100% P-polarized.

The metal-dielectric waveguide (MDW) is similar to the SP structure but contains an additional layer of dielectric (for example silica) or polymer on top of the Ag surface (Figure 2, bottom). This layer results in additional optical modes and additional cones of emission [55–56]. The dielectric thickness determines the presence of different modes and their polarization. While difficult to discern from Figure 2 the S-polarized emission displays a more narrow angular distribution which suggests lower optical losses than for P-polarized coupled emission. These simple structures were found to be very efficient in collecting about 90% of the energy from surface-localized fluorophores [57–59] and to provide surface selective detection [60].

Figure 3 shows structures with more complexity, but which also provides additional opportunities in CEM. The top structure consists of multiple layers of silica (SiO_2 , $n = 1.46$) and silicon nitride (Si_3N_4 , $n = 2.15$). This structure is called a one-dimensional photonic crystal (1DPC) because it is heterogeneous in one direction. The 1DPC with certain thicknesses of these layers displays a photonic band gap, which is a range of wavelengths that cannot exist in the structure [61]. By modification of the top layer thickness of the 1DPC the optical energy can be trapped in this top layer and at the dielectric-air interface. These modes are known as Bloch surface waves (BSW) [62–63]. We recently reported that fluorophores near the top surface of the 1DPC can couple with the BSWs and with the internal optical modes of the 1DPC resulting in coupled emission at different angles and polarizations [64–66]. BSW modes are S-polarized and have a very narrow resonance. As a result the emission coupled to the BSW is also S-polarized and has a very narrow angular distribution. The P-polarized emission is due to coupling to modes inside the 1DPC.

The final structure is a Tamm structure (Figure 3 bottom). This structure is comparable to a 1DPC except for a different thickness for the top dielectric layer which is then coated with a 45 nm Ag layer. The unique feature of Tamm structures is that optical mode exists with light at normal incidence from either side of the sample (0 or 180) [67–68]. As a result coupled emission can occur directly normal to the surface, frequently with additional off-angle

modes [69–70]. Since these angular distributions for all the structures were measured without the effect of refraction (Figure 4) they display the same directionality which must exist under the MLSs, and which would propagate into an optically coupled medium. The use of several MLS (Figures 2 and 3) provides a test of the generality of this proposition.

Back focal plane and front focal plane imaging

CEM and out-of-focal plane (OFP) imaging uses concepts from both FFP and BFP imaging. These concepts and optical configurations can be confusing and need a brief overview. To avoid confusion it is valuable to define the terms. The FFP of an objective exists at a close distance from the front of the objective, and the BFP is located inside most objectives (Figure 5). The right side of Figure 5 shows the location of images of the FFP and BFP. One of the more common uses of microscopy is imaging of cells which are labeled with fluorescent probes. In these experiments the goal is to measure the spatial distribution of fluorescence in the x-y plane, which is perpendicular to the optical z-axis. This is accomplished using an objective which focuses the magnified image at a location where it can be observed, which is called the front focal plane (FFP) image of the objective (Figure 5, top). If there is a convex lens behind the objective Figure 5 (top) the emission from every spot on the sample is focused to a unique location in the magnified image at the FFP. The BFP image provides different information. All the emission from the entire FoV is resolved according to the angle at which the light enters the objective. The BFP image does not reveal the spatial origin of the emission within the FoV. In many published papers the BFP concept is represented in the middle panel of Figure 5. Light which enters the objective at a given angle arrives at the detector at a specific distance from the center of the optical z-axis. However this schematic omits some optical elements which are used when BFP optics are configured. Additionally, the simplified BFP schematic in Figure 5 prevents us from consideration of other positions for the detector because this schematic suggests the same image will be observed at any location behind the objective, and there is no way to obtain a FFP image using this schematic for BFP imaging.

At present almost all microscope objectives are “infinity corrected” (IC), and this term has resulted in some confusion. The term “infinity corrected” is often represented as meaning the light exits the objective as a parallel beam (Figure 5, middle). This is incorrect and light emerging from the objective and forms an image at an infinite distance, where of course it cannot be observed. An IC objective requires an additional lens, usually called a tube lens, to collect the emission and focus it on the array detector (Figure 5, bottom). Depending on location of the detector images of either the FFP images or BFP images can be observed but physical constraints in commercial microscopes prevents movement of the detector to the actual BFP which is usually inside the objective.

Infinity-corrected objectives have a favorable optical property that simplifies the design and use of microscopes. There exists a section in the optical path where other optical components like filters, polarizers or beam splitters, can be inserted into the optical path without significant distortion or loss of spatial resolution. With an IC objective the light rays are collected by a tube lens, which redirects the light towards the detector. We used the optical configuration in Figure 6 which allowed insertion of a beam-splitter without

distortion of the image. Our configuration allowed detectors to be located at the FFP and the BFP image planes. Alternatively, one detector could be moved from the FFP to the BFP along the optical axis to obtain either type of image.

There is another effect which complicates the interpretation of BFP images. As shown in Figure 2 emission from fluorophores on a glass slide occurs over a wide range of angles. In all the other structures shown in Figures 2 and 3 the light emerging from the MLS is distributed at one or two angles around the z-axis. Therefore we need to understand how these sharp angular distributions appear in the FFP and BFP images.

Examples of BFP Imaging

Prior to a discussion of OFP imaging it is valuable to understand the BFP images observed with the multi-layer structures in Figures 2 and 3. These images were obtained using a continuous film of Nile Red (NR) in PMMA. The size of the film is larger than the FoV of the objective (Figure 7), but a focused laser beam is used to illuminate a small spot which provides a point source of light about 2 microns in diameter. If this sample was observed in the usual way, at the FFP of the objective, the image would be a single spot with a similar diameter (of 2 microns) to that of the illumination spot (not shown). Dramatically different images are observed at the BFP which can be understood by comparison with the angular intensity distributions in Figures 2 and 3. The easiest image to explain is the Ag-coated glass sample (SP). It is known that fluorophores near the top silver film create surface plasmons, and due to wavevector matching considerations these plasmons can only propagate at a single angle down through the glass substrate. The emission occurs at a single angle θ from the z-axis (Figure 2 middle) resulting in a cone of emission with the same angle θ (Figure 7, middle left). Similar reasoning can be used to interpret the MDW BFP image in Figure 7 (Figure 7, middle right). Addition of a thicker dielectric layer on the top SP structure allows the MDW structure to support two or more optical modes [71–72]. These modes have different field intensity distributions in the structure and radiate through the glass substrate at different angles (Figure 2 bottom), which appear as two rings in the MDW BFP image. These rings have different polarizations (Figure 2) which can be seen by insertion of a polarizer into the emission pathway (not shown). The polarization and diameter of the rings are generally related to the different optical modes sustained by the multilayered structures.

The 1DPC and Tamm BFP images in Figure 7 are less well defined which may be the result of multiple layers with imperfections at the interface between the layers, or the result of the wide emission spectrum of NR and the dependence of the coupling angle on wavelength. Nonetheless, general features of the images can be understood. For the 1DPC, most of the emission couples into the BSW mode, with a very sharp angular distribution (Figure 3), which is S-polarized (not shown). The dominant coupling into this mode results in the single bright ring in the BFP image in Figure 7. The BFP image of the Tamm structure contains a central peak which was not observed with the other structures. The central peak confirms that a Tamm structure can result in coupled emission which is directed along the z-axis. For any of the MLSs if the numerical aperture (NA) of the objective is known the diameters of these rings can be used to calculate the angles of the coupled emission.

The most complex BFP image is obtained using a plain glass slide (Figure 7, top right). In this case the emission is distributed over a wide range of angles and light is detected at all distances from the center of the image. The change from a darker to a brighter ring at the critical angle demonstrates that the more emission from a fluorophore on a glass slide is coupled into the glass above the critical angle (Figure 2, top right) and can only be observed with an immersion objective.

Defocused Imaging

The schematic in Figure 1 suggests that the sub-surface fields could be measured by moving the focal plane of the objective (Figure 8). The objective focal plane can be adjusted to be either above (+Z) or below (−Z) the plane in which the point sources are located. Displacement of the objective downwards (under focus) results in rings typical of SPCE (Figure 9). Similar rings of emission are observed when the focal point is above the metal and polymer films (over focus). Emission from fluorophores which are more than a couple of microns above the metal film is not expected because light incident from the RK direction cannot create surface plasmons and is reflected, and surface plasmons are created by the near-field interactions for fluorophores close to the metal film [73–74]. Similar results were observed for the MDW structure that is under focusing resulted in rings of emission (Figure 10). These rings were not observed with over focusing where the detected emission decreased almost to zero. In the MDW structure the fluorophores are separated from the Ag film by about 320 nm layer of silica. At this distance there are no fluorophore-metal near-field interactions and free-space emission which is not coupled to the MDW is reflected. The sharp decrease in total intensities with over focusing (+Z) was clipping or blocking the emission at high angles due to some barrier in the objective. This interpretation is supported by the over focusing results for the SPCE structure (Figure 9) where coupled emission angles are near 45°, as compared to the larger angles near 60° for the MDW structure (Figures 2, 3 and 10). These rings obtained by defocused imaging are probably due to plasmons. However, rings have also observed for fluorescent and non-fluorescent particles in the absence of any metal or dielectric surface [75–76] and the ring dimensions may be an optical property of the particular objective or due to Fraunhofer diffraction by a circular aperture. At present we are exploring confocal fluorescence detection using the approach shown in Figure 8 to detect sub-surface optical fields.

FFP and BFP imaging of a continuous fluorescent film on glass and an SP structure

The previous BFP images were obtained using a focused laser beam to illuminate a MLS. It is informative to compare the FFP and BFP images when a large continuous fluorescent film is excited on a plain glass or an SP structure (Figure 11). We used a continuous NR-labeled film which covered the entire FoV of the objective (Figure 11). The incident laser beam was expanded to cover the whole FoV of the objective. When the film is on glass the FFP image shows uniform brightness as is expected for a homogeneous fluorescent film. The BFP image from this continuous fluorescent film looks almost exactly the same as was observed with illumination of a small spot in the center of the objective (Figure 7, upper right). The similarity of the BFP images for a single illumination spot and illumination of entire FoV on a glass substrate, demonstrates that the BFP images contain no information about the spatial distribution of fluorescence in the x-y focal plane. Similar results are obtained using the SP

substrate, which is the Ag covered glass slide. In this case the FFP image also shows uniform brightness, but less intense due to light losses using the silver film. The BFP image is a ring of emission, the same result as a fluorescent spot (Figure 7). This result indicates that the coupled emission emerging from the SP structure occurs at the same angle, irrespective of location in the FoV. These results led us to question if the FFP and BFP images can be combined to obtain spatial and angular resolution in a single image.

Imaging of fluorescent nanobeads

In order to investigate the correlation between the FFP and BFP images we needed samples with a known distribution of emission at the FFP. This was obtained by using structures which were drop coated with dilute suspensions of NR-labeled polystyrene spheres. Each of these nanobeads (NBs) contained numerous fluorophores and single NB could be easily identified. In contrast to single fluorophore detection, the NBs did not blink and did not photobleach significantly during the experiments. The samples with surface-bound NBs within the FoV were examined using the optical configuration shown in Figure 12. This configuration functioned like the schematic shown in Figure 6, but allowed continuous movement of the CID between the image of the front and that of the back focal planes. As seen in the ray tracings in the lower part of Figure 12, there must be a continuous change in the image from two spots in the FFP image to a single ring in the BFP image. We reasoned that the out-of-focal plane (OFP) images will contain information about both the spatial and angular distribution of emission from the sample. To the best of our knowledge out-of-focal plane (OFP) images have not been previously reported.

We examined a SP structure with two NBs within the FoV. Initially we moved the objective as shown in Figure 8 and expected to observe 2 fluorescent spots merging into a single SPCE ring. The images of each bead showed a ring as expected for SPCE (Figure 13). However, we were unable to move the objective too far, which may be due to beam clipping in the objective. Also, the actual BFP of a typical objective is inside the objective. The NB emission decreased and became un-observable for large displacements of the objective. We were surprised that the NB images remained at the same distance from each other as the objective was moved because we knew the two rings had to merge into one ring in the BFP. In fact, this was the observation which led us to realize that the commonly used schematic for BFP imaging (Figure 5) did not provide an adequate description of BFP optical system.

The results were dramatically different when the detector, instead of the objective, was moved between the FFP and BFP image locations. When the detector was displaced the rings from the NBs became larger (Figure 14, at 50 mm). The distance in mm refers to the displacement of the CID from the FFP image plane in Figure 12. Importantly, as the detector was moved further, the rings grew larger they became closer together, began to overlap (175 mm) then nearly merged into a single SPCE ring (250 mm). Similar results were obtained for all the structures except for plain glass slides. The images of three NBs on the 1DPC became larger as the objective was moved but the distances between the spots remained the same (Figure 15). When the CID was moved the spots became larger and closer together (75 nm), overlapped (175 nm) and merged into a single ring (250 nm) (Figure 16). Similar results were obtained using the MDW structures (Figures S3–S4). For the Tamm structure

the two spots in the FFP image merged into a single central spot in the BFP image (Figure S5). Rings are not observed with the Tamm structure because the emission occurs directly along the z-axis. The weak outer ring in the Tamm BFP image is due to coupling into off-axis modes of the structure [69–70].

As a control experiment we used NBs on a plain glass cover slip (Figure 17). Moving the objective resulted in more diffuse spots but no change in location. Movement of the detector resulted in diffuse circles of nearly uniform brightness (Figure 18) because, without a MLS, the emission at all angles can enter the objective at all angles and thus result in a circle of uniform brightness in the BFP (250 mm). These results show that the OFP images on glass containing information about the spatial distribution of emission in the x-y FoV. However, it will be easier to count particles with the MLSs because they provide narrow rings of emission rather than diffuse spots. Figure 19 demonstrates the increased ability to count NBs when using a MLS. The SP structure was coated with a higher concentration of NBs. The multiple rings corresponding to about 20 NBs can be readily counted in the OFP images with the detector at the 25 mm location. The ability to count the number of NBs, within such OFP images may be useful in devices such as Figure 1 because a FFP image may not be available without the use of external optical components. We expect CEM and OFP images to be used in multiplex assays using NBs of different colors, or NBs which emit at multiple wavelengths. Algorithms have already been developed to count the number of rings from scattered light where the objects are not located in a single plane and the rings have different diameters [77]. A ring counting analysis may be less demanding in CEM because most NBs will be close to the MLS.

All the results presented above were obtained using spherical NBs. The OFP images of less symmetric objects are not easily predictable. As one example, Figure 20 shows images of light from a NR-labeled nylon nanofiber about 200 nm in diameter located on the SP structure. The FFP image is the expected straight line (Figure 20). As the detector is moved the OFP images take on the appearance of a row of overlapping circles where the circles become larger and more overlapped. The BFP image displays an emission cone typical of a SP structure. In the final BFP image the multiple rings collapse into a single ring. However, there are two additional features, which are straight lines, tangential to the rings and perpendicular to the long axis of the nanowire. These straight lines were initially unexpected and puzzling. After searching the literature we found that straight lines were observed with light scattering or coupled scattering from metallic nanofibers (with no fluorophores) [78–80]. These straight lines have been attributed to leaking from a waveguide at a well-defined k-value (wavevector) after some guiding has occurred. This result demonstrates the different information which can be obtained by measurement of the OFP images.

In all the above defocused imaging experiments the objective was moved distances up to 9 μm . In contrast, for the OFP experiments the CID was moved by distances up to 250 mm. This dramatic difference in distances is probably related to the high magnification along the z-axis, which is typically near the square of the magnification in the x-y plane [81–82]. This topic is rarely mentioned in the scientific literature, and not mentioned in books on optical microscopy [83]. This topic is complex and the z-axis magnification may not be the same for

different displacements of the detector and may not be constant over the entire FoV. These concepts are presently being investigated and will be the subject of future reports.

Discussion

It is valuable to compare our results with previous publications on imaging using SPCE from metal-coated slides for sensing assays [84] and in some cases for imaging [85]. In these previous publications a glass prism is used to observe the coupled emission, which always occurs above the critical angle. For these sensing and imaging experiments only a small angular segment of the emission cone is detected. Image formation was accomplished using laser-scanning or stage-scanning confocal microscopy. In contrast to these previous reports we do not attempt to form a focal plane image, but accept the emission cone as the primary form of the signal. This allows us to collect all of the coupled emission and to eliminate the need for focusing optical components.

Our CEM concept is distinct from previous publications on fluorescence imaging with CMOS cameras or cell phones. The majority of published reports on low cost fluorescence imaging with cell phones require external lenses and other external components to collect and focus the fluorescence [86–90]. Lens-free detection of fluorescent nanobeads down to 10 μm diameter has been accomplished, but these measurements used a fiber optic faceplate and computational reconstruction [91].

CEM should not be confused with other reports of lens-free cell imaging [92–94]. These reports do not use fluorescence, but instead use partially coherent illumination and collection of images of the scattered light. Each location in the sample contributes to the scattered light intensity measured at all pixels, resulting in an incomplete hologram of the sample. Extensive computations are required to reconstruct an image of the sample. The use of scattered light does not have the opportunities for selective observation provided by the numerous available fluorescence probes.

The usefulness of CEM devices will be facilitated by its large field-of-view (FoV). As mentioned in Introduction, with traditional magnifying optics the field-of-view (FoV) decreases as the magnification increases. For example, the field-of-view (FoV) (diameter across the image) for a typical microscope with 10X magnification is about 2 mm. For 40 and 100 $\times$ magnifications the FoV decreases to about 450 and 180 μm , respectively. Only a small fractional area of a slide can be viewed at one time. At high magnification, careful optical engineering is needed to maintain spatial resolution and color-correction, which limits the size of practical microscope objectives. The FoV with CEM can be as large as needed and is limited only by the dimensions of the CMOS chips which range from 1 $\times$ 1 mm to imaging areas which are many cm wide. In CEM devices there is no magnification and the spatial resolution is the same over the entire CEM area. As an example an entire printed DNA array could be imaged by a CID (Figure 21) without the need for point by point scanning of the slide. CEM devices are most likely to be used in targeted DNA testing where a smaller number of spots are present as compared to whole-genome testing.

The results in this paper suggest that CEM can be adapted to a wide range of bioaffinity assays, immunoassays and flow cytometry assays [95–98]. Many of these assays are based on fluorescent NBs [99–102]. A wide range of particle sizes and wavelengths are commercially available, including multi-color NBs for multiplex assays. These tests are often designed for use of a disposable cartridge which contains a porous polymer strip designed for lateral flow immunoassays (LFA) [103]. A small sample of bodily fluid (blood, urine or saliva) is placed on an open well and is carried across the test strip by capillary action. The target species are trapped on the strip at locations where the capture molecules are located. A similar approach can be used with CEM devices (Figure 21). Microfluidic devices are often based on PDMS which readily adheres to glass surface without leaks at the PDMS-glass interface [104–105].

Our investigation of coupled-emission microscopy provides an understanding how to create a new generation of devices for sensing, biomedical research and diagnostics self-testing. This evolutionary process is being enabled by rapid developments in electronics, imaging and computational power. To illustrate the future use of CEM several alternative optical configurations are shown in Figure 22. These hypothetical devices illustrate how devices could be made which are small, robust and without the use of free-space optics. Excitation could be accomplished with edge illumination of a waveguide, which can be assisted by grating, lines, or particles at the site of illumination. The evanescent field propagating across the structure can be the source of excitation of the fluorophore. Another approach is illumination from below the MLS. All the structures shown in Figures 2 and 3 display angle-dependent resonances for coupling incident light. Both of these optical configurations reduce the ubiquitous problem of rejecting the scattered incident light which is present in many measurements of fluorescence. These optical configurations would provide surface-localized excitation and thus reduced emission from the bulk sample which is not immediately adjacent to the surface. The use of MLS provides angular and wavelength separation starting at the origin of the emission without the use of a separate dispersion element. An important advantage of CEM devices similar to those in Figure 22 would be a complete absence of interfaces which include free-space propagation of light and total internal reflection above the critical angle.

Instrumentation for fluorescence detection and imaging still use a multitude of separate optical components to collect a fraction of the omni-directional emission, and to redirect and focus the free-space emission. Even the smaller devices used for point-of-care diagnostics often retain the use of multiple optical components to manipulate the free-space emission. We believe the cost and complexity of fluorescence instrumentation can be greatly reduced by using CEM with photonic or plasmonic structures. The use of our simple multi-layer structures, which do not require top-down nanofabrication, will allow low-cost fabrication of large areas structures up to the size of slides or microwell plates. Combining these structures with rapidly evolving CMOS imaging detectors can result in new classes of lens-free devices for fluorescence detection. The development of low-cost CEM devices is made possible by the rapid evolution of CMOS array detectors. Several years ago CCD cameras were regarded as state-of-the-art, and CCD cameras still have some advantages in low-light and low frame rate applications. However, CMOS devices have several advantages over CCDs and are rapidly replacing CCDs for most applications. CMOS detectors consume 100-fold less

power, uses lower voltages and generates less heat [106–108], which may become important with nearby biomolecules. The low power consumption and fast frame rates allows video cameras to be included in most cell phones. Because of the large consumer markets, CMOS cameras with the electronic controls and with over 500×500 pixels can be purchased for under \$10. The average cost of a separate CMOS array detector is now less than \$2.00 [109]. CMOS detectors are much less expensive than CCD detectors because CIDs are made in the same foundry as other semi-conductor devices. CCD detectors have to be made in a separate foundry and then combined with the other electronic components. Our CEM concept will provide 1- to 1-imaging. The ultimate spatial resolution will be comparable to the pixel size. The pixel size has been decreasing rapidly and the latest CMOS cameras have pixel dimensions of less than $2 \mu\text{m}$ across. Sub-pixel spatial resolution is possible using interpolation and/or fitting procedures [110]. The pixel size is expected to decrease below $1 \mu\text{m}$ and will not limit the spatial resolution which is likely to be within 2-fold of a diffraction limited microscope.

In summary we have described a method to image the optical fields below the surface of multi-layer photonic or plasmonic structures. These images are obtained with the imaging detector positioned at locations between the FFP image plane and BFP image plane. The OFP images represent the optical field which are directly under the MLS and will be seen with a multilayer photonic or plasmonic structure which is placed on a CMOS imaging detector. This capability will facilitate the development of CEM devices for medical diagnostics and biological research. Additional research is needed to fully understand how the structural features of the observed sample contribute to features in the OFP images.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Key Basic Research Program of China under grant nos. 2013CBA01703, 2012CB921900, 2012CB922003, the National Natural Science Foundation of China under grant nos. 61427818, 11374286, Science and Technological Fund of Anhui Province for Outstanding Youth (1608085J02). This work was also supported by grants from the National Institute of Health, GM107986, EB006521, EB018959 and OD019975. The authors thank the reviewer(s) for their detailed comments which clarified the description of CEM

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Figure 1. Schematic of coupled emission microscopy with a MDW and a CID. In applications the MDW will be in direct contact and/or optically coupled with the CID. The emission filter can be a thin film of a dielectric containing a dye which absorbs the incident light. Alternatively, the supporting dielectric can also be the emission filter.

Figure 2.

Substrates used for measurements of coupled emission from RhB in a polymer film. All angular distributions are with normal incidence from the top of the structure (180°). Emission angles are measured from below the structure starting at the Z-axis at 0°

Figure 3. Additional substrates used for measurements of coupled emission from RhB in a polymer film. All angular distributions are with normal incidence from the top of the structure (180°). Emission angles are measured from below the structure at 0° starting at the Z-axis.

Figure 4. Schematic of the MDW structure with a continuous fluorescent film and the optical geometry.

Figure 5.

Optical configurations for a microscope objective. Top, typical objective for optical microscopy. Middle, frequently used representation of BFP imaging. Bottom, correct representation of BFP imaging. We note that the light propagation angles change at every glass-air interface inside the objective. For simplicity these changes are not shown in Figure 5.

Figure 6. Optical configuration used for BFP, OFP and FFP images of fluorescent films, NBs and nanowires. 100 × objective is used for fluorescent films and NBs, a 60 × objective is used for nanowires imaging.

Figure 7. BFP images for spot illumination of NR in 25 nm of PMMA on structures shown in Figures 2 and 3. The top left panel shows the optical geometry.

Figure 8.
Optical configuration used for objective defocused measurements.

Figure 9. Over focus (left) and under focus (right) of NR in 40 nm thick PMMA on a 45 nm Ag film (SP structure). The distance, d , is the distance of the objective focal point to the top of the metal film.

Figure 10.

Over focus (left) and under focus (right) of NR in a MDW with 320 nm thick PMMA on a 45 nm Ag. The distance, d , is the distance of the objective from the FFP.

Figure 11. FFP and BFP images of a continuous film of NR in PMMA on the glass (middle column) and SP structure with 45 nm Ag (right column). The fluorescent film completely covered the FoV of the objective and the laser beam was focused in the center of the objective.

Figure 12.

Optical configuration for OFP imaging and sub-surface field imaging. The distance between FFP and BFP is 250 mm with a $100\times$ objective. Schematic is not drawn to scale.

Figure 13.

Images of two NR doped NBs on the SP substrate (SP, top left) with objective is moved down (under focus as shown in Figure 8) from the original focal plane (silver surface). The yellow arrows point to the NBs.

Figure 14.

Optical images of the NR-labeled NBs on the SP substrate in Figure 13 with the detector moving down from the focal plane (Figure 12).

Figure 15.

Optical images of the NR-NBs on a 1DPC with the objective is moving down from the original focus position (1DPC surface) by distance labeled in each image.

Figure 16.

Optical images of the NR doped NBs on the 1DPC shown in Figure 15 with the detector moving down from the focal plane (Figure 12).

Figure 17.
Optical images of NBs on glass with the objective is moving down from the focus position by distance labeled in each image.

Figure 18.

Optical images of the NR doped NBs on glass as shown in Figure 17 with the detector moving down from the front focal plane (Figure 12).

Figure 19.

FFP, OFP and BFP images of a cluster of NBs on the SP structure. $60\times$ oil objective with NA of 1.49 is used. Emission observed through a 560 nm edge filter.

Figure 20.

Optical Images of a NR-labeled Nylon-6 nanofiber with a 200 nm diameter. The detector is moved down for the FFP (0 mm) to the BFP.

Figure 21.
Additional applications for CEM to printed arrays and lateral flow immunoassays.

Figure 22.
Alternative configurations for detecting NBs using CEM using TIR or edge incidence for excitation.