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Entangled-Photon Microscopy

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IN HONOR OF THE SIXTIETH BIRTHDAY OF PROFESSOR JAN PEŘINA, COLLEAGUE AND FRIEND OF 25 YEARS

All forms of microscopy that have previously been developed rely on classical sources of light, which are relatively noisy by virtue of the photons arriving randomly in time and position. We propose a new technique of two-beam optical microscopy using a nonclassical light source comprising entangled photon pairs generated by the process of nonlinear optical parametric downconversion. Entangled-photon microscopy (EPM) has the potential for providing a substantial improvement over confocal laser scanning fluorescence microscopy (CLSM) as well as over two-photon laser scanning fluorescence microscopy (TPLSM). The latter technique relies on the proportionality of the two-photon absorption rate to the square of the optical photon-flux density, and makes use of a highly focused optical beam to localize the region from which fluorescence is observed. However, because the arrival of pairs of photons is accidental in TPLSM, relatively large values of the photon-flux density are required to achieve photon coincidence and hence absorption. This can lead to undesired phototoxicity and photobleaching of the specimen. EPM, in contrast, relies on the presence of highly correlated photon pairs, which enhances the two-photon absorption rate significantly and enables lower values of the photon-flux density to be used. The process depends linearly, rather than quadratically, on the photon-flux density. Further, the EPM interaction region can be controlled by the use of two beams, providing increased flexibility in the selection of desired regions of a specimen, and leading to enhanced axial and lateral resolution.

1. INTRODUCTION

For several hundred years, the microscope has allowed us to peek into tiny domains invisible to the naked eye. Conceived by Galileo in the early 1600's, and perfected by Abbe in 1873 [1], this remarkable instrument continues to serve us nobly today.

In recent years, there has been a host of improvements in microscopy, resulting from significant advances in light sources, optical components, detection systems, and computational methods. Moreover, it has become possible to select for measurement a variety of optical attributes of a specimen, aside from the usual features of absorption and reflection [2]. One of the most useful of these turns out to be optical fluorescence, either of the specimen itself or of an externally provided selective marker. In recent years, wide-field (WF) fluorescence microscopy

has become a valuable technique for enhancing both the sensitivity and specificity of microscopic imaging.

A particularly important advance in microscopy arrived with the first confocal microscope, conceived by Minsky in 1957 [3]. As Minsky put it, confocal microscopy offers a substantial improvement in resolution over conventional microscopy by providing a method "for rejecting all scattered light except that emanating from the central focal point, *i.e.*, the illuminated point of the specimen."

The combination of fluorescence imaging and confocal microscopy has become particularly powerful, and has literally ushered in a new era of microscopy [4,5]. Confocal laser scanning fluorescence microscopy (CLSM) provides a substantial advantage over WF fluorescence microscopy by dramatically reducing out-of-focus background light from the specimen. CLSM permits thin *optical* sections to be imaged from within an intact living fluorescence-labeled specimen, without the necessity of slicing the preparation into thin *physical* sections. A three-dimensional distribution of fluorescent markers can readily be constructed from a collection of CLSM optical sections, with diffraction-limited spatial resolution [6].

In the never-ending quest for improved measurement techniques, several alternatives to CLSM have been recently considered. One such scheme is two-photon laser scanning fluorescence microscopy (TPLSM) [7-9], which has a number of salutatory features in comparison with CLSM, as discussed in Section 2. This technique makes use of a highly focused optical beam to localize the region from which fluorescence is observed and relies on the proportionality of the two-photon absorption rate to the square of the optical photon-flux density [10]. This quadratic behavior, which is the hallmark of TPLSM, is a result of the accidental arrival of pairs of photons emitted by classical light sources [11]. TPLSM therefore generally requires the use of high photon-flux-density optical sources, often femtosecond pulsed lasers, to insure that the two photons have a significantly substantial probability of arriving simultaneously and effecting an absorption. However, this can result in phototoxicity and photobleaching of the specimen.

Classical light sources, both natural and artificial (e.g., skylight, sunlight, starlight, incandescent light, fluorescent light, and laser light), are intrinsically noisy as a result of these random photon arrivals [12]. In recent years, nonclassical (quantum) sources of light have been developed in which the intrinsic noisiness is reduced in one way or another [13-18]. This attendant reduction of noise can, if harnessed properly, improve the fidelity of optical microscopy. As discussed in Section 3, entangled-photon sources emit pairs of photons nearly simultaneously [19], so that two-photon absorption has the potential for taking place at substantially lower light levels than in the case of accidental arrivals [20,21].

We propose a scheme called entangled-photon microscopy, which makes use of nonclassical light to reduce the photon-flux density levels at which two-photon absorption occurs, and thereby to avert phototoxicity and photobleaching. Because each photon of the pair comes from a different optical beam, the volume of entanglement is adjustable thereby providing an additional measure of control for selecting the location and size of the desired absorption region. The relative advantages of EPM with respect to TPLSM are highlighted in Section 4.

2. TWO-PHOTON LASER SCANNING FLUORESCENCE MICROSCOPY

Pawley [5] and Sandison *et al.* [6] have summarized some of the performance limitations associated with CLSM, such as the complexity of confocal spatial-filtering optics and the restrictive modes in which detection can be achieved. One of the more promising alternative approaches is two-photon laser scanning fluorescence microscopy (TPLSM). Kaiser and Garrett

[22] were the first to observe two-photon fluorescence, in 1961. Though Hellwarth and Christensen [23] used a form of two-photon optical microscopy in 1974, the first conception of a two-photon laser microscopy system (in a cavity configuration) appears to be due to Sheppard and Kompfner [7]. More recently, the technique has been implemented in a traveling-wave configuration by Denk *et al.* [8]. TPLSM has been found to be useful for dynamic imaging in a rapidly growing number of biological systems [24].

The technique operates in the following manner: two photons that find themselves in the vicinity of a fluorescent probe [25] linked with the specimen, and that arrive within a time shorter than its lifetime [11], can effect an absorption. The emission of a single fluorescence photon from the probe is then detected using conventional means. The result is a quadratic dependence on the incident photon-flux density ϕ, of the two-photon absorption rate and thereby of the emitted fluorescence photon rate [22]. The distinctive characteristics of TPLSM derive principally from the realization of this quadratic behavior in space, which results in a sharpening of the active focal region of the excitation light beam. This, in turn, results in an enhancement of resolution in the axial direction and a reduction of background light arising from out-of-focus fluorescence. TPLSM therefore bears some similarities to one-photon (ordinary) CLSM.

Because the arrivals of classical photons are purely random, however, relatively large incident optical photon-flux densities are required for a photon pair to accidentally arrive within a narrow time window (the intermediate state lifetime of the specimen), and thereby to result in two-photon absorption. Optical excitation in the form of ultrashort (femtosecond) pulses (usually generated by mode-locked lasers) is therefore generally required, giving rise to a peak photon-flux density that is sufficiently large so that two-photon absorption can occur, while maintaining an average photon-flux density that is sufficiently small so as to be tolerable to a biological specimen.

We summarize some of the salutatory features of two-photon microscopy with respect to confocal laser scanning fluorescence microscopy [9,26]:

- The technique offers excitation-engendered depth discrimination, similar in magnitude to that achievable in CLSM, but obtainable without the need for confocal spatial-filtering optics. The background light arising from out-of-focus fluorescence is concomitantly reduced.
- The emitted fluorescence radiation need not be focused, descanned, or passed through special optics, thereby permitting detection to be carried out in a broader variety of configurations than with CLSM. This can be beneficial for imaging in media with certain special properties. An example is provided by brain tissue, in which light is scattered more strongly at shorter wavelengths than at longer wavelengths. Longer wavelength excitation light can therefore penetrate more deeply into the interior of a sample while the shorter wavelength emitted fluorescence can be detected over a relatively large solid angle.
- Systems can be designed to make use of markers other than optical fluorescence to signal the presence of two-photon excitation. One such scheme, recently developed by Denk [27], is called two-photon scanning photochemical microscopy (TPSPM). In one particular implementation, local two-photon absorption causes a photoactivatable probe to release neurotransmitter molecules; the binding of the free neurotransmitter to nearby ligand-gated ion channels is then detected by the induction of cellular ionic current flow. This scheme has been used to generate images of acetylcholine receptors on a cell membrane using caged carbamoylcholine as the probe [27]. Other implementations, such as the release of photon-activatable reagents, have also been considered.

3. TWO-PHOTON ABSORPTION OF NONCLASSICAL LIGHT

The essential distinction between two-photon absorption from a classical photon source and from an entangled-photon source is captured by a simple probabilistic model that treats the photons as particles [21].

The absorption process is considered to consist of two steps: the first photon initiates a transition from the lower (ground) state of a material system to a virtual (intermediate) state and the second photon brings about a transition to the final state. For randomly arriving photons from a classical source of light, the probabilistic analysis yields a transition rate R_r (absorptions per sec) that depends only on the material's single-photon cross section σ and the virtual-state lifetime τ. The resulting two-photon absorption rate is $R_r = \delta_r \phi^2$ where the two-photon quadratic cross section is $\delta_r = \sigma^2 \tau$ [28].

We now turn to correlated photon pairs from an entangled-photon source [29] that arrive at the absorbing medium with a pair flux density $\phi/2$ (photon pairs/cm^2-sec). In this case, the absorption rate of the material depends on the probability $\xi(T_e)$ that the two photons emitted within the time T_e arrive within τ and the probability $\zeta(A_e)$ that the two photons emitted within the area A_e arrive within σ. Thus, the correlated two-photon absorption rate is $R_e = \sigma_e \phi$ with cross section $\sigma_e = \frac{1}{2}\sigma\xi(T_e)\zeta(A_e)$. This rate must be supplemented by that representing the accidental arrival of pairs within τ and σ (calculated above for classical light), resulting in an overall two-photon absorption rate *R* given by [21]

$$R = R_e + R_r = \sigma_e \phi + \delta_r \phi^2 . \tag{1}$$

It is clear that correlated two-photon absorption dominates random two-photon absorption for small values of the photon-flux density. The critical photon-flux density ϕ_c at which the two processes are equal is

$$\phi_c = \sigma_e / \delta_r . \tag{2}$$

For $T_e << \tau$ and $A_e << \sigma$, $\xi(T_e)$ and $\zeta(A_e)$ are unity, yielding $\sigma_e = \delta_r / 2\sigma\tau$. In the experimentally relevant case in which $T_e >> \tau$ and $A_e >> \sigma$, the probability functions are $\xi(T_e) = \tau / T_e$ and $\zeta(A_e) = \sigma / A_e$, yielding $\sigma_e = \delta_r / 2A_e T_e$ so that $\phi_c = (2A_e T_e)^{-1}$.

A detailed quantum-mechanical calculation of the two-photon linear absorption rate with entangled light has recently been carried out [21]. The results confirm Eq. (2), but they also reveal the presence of nonmonotonic variations in the absorption rate R_e as a function of both the entanglement time and the path-length delay between the two excitation beams. An important feature of these variations is a significant enhancement in the absorption cross section σ_e for certain parameter values. The quadratic two-photon absorption cross section δ_r for classical light, in contrast, exhibits no such enhancement [30].

4. ENTANGLED-PHOTON FLUORESCENCE MICROSCOPY

Entangled-photon fluorescence microscopy (EPM), illustrated in Fig. 1, makes use of a light source comprising twin beams of quantum-mechanically entangled photons, which exhibit photon-pair occurrence times that are highly correlated. Such beams may be generated by a nonlinear optical process such as spontaneous parametric downconversion [31].

Because energy is conserved in the process, the entangled photons are produced nearly simultaneously and each has a wavelength longer than the original. Momentum is also conserved, resulting in a one-to-one correspondence between the direction of travel of a photon in one beam and the direction of its matching photon in the other beam [32]. Because they share the energy and momentum of the original photon, the twin photons are said to be "entangled" with each other [17,18].

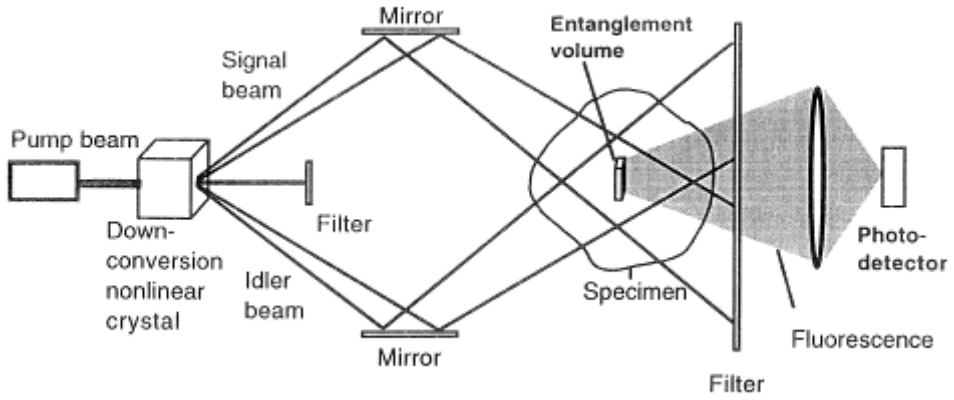

Fig. 1. Entangled-photon microscopy.

Provided that the critical photon-flux density ϕ_c is sufficiently large, entangled-photon microscopy has a number of features that can render it superior to two-photon laser scanning microscopy. We detail some of these advantages, which ultimately stem from the quantum-mechanical correlation of the photon pairs:

- EPM operates at reduced light levels relative to TPLSM, thereby minimizing undesirable phototoxicity and photobleaching; this can be particularly important for sensitive biological specimens. Low light levels also minimize shifts of the natural energy levels of the sample, such as those that arise from the Stark effect.
- The two excitation beams can be directed by optical components to cross each other with a variable relative path-length delay, thereby permitting the position in the specimen at which the entangled photons simultaneously arrive to be selected. The observation location in the specimen can therefore be changed or systematically scanned (both axially and laterally) by simply altering a collection of optical components. Moreover, the entangled photons can be arranged to intersect the specimen in a variety of configurations, including at multiple locations. The components directing and guiding the beams can include mirrors, lenses, prisms, gratings, holographic components, graded-index optical components, optical fibers, and optical-fiber components. TPLSM generally relies on mechanical scanning.
- Entanglement-time tuning can be combined with path-length delay tuning to create small and adjustable regions of the specimen in which photon pairs arrive nearly simultaneously [21],

providing enhanced resolution (width of region of photon simultaneity) and localization (decay of region of simultaneity with distance), in both the axial and lateral directions. In EPM, the resolution and localization are therefore determined by the adjustment of optical components whereas in TPLSM they are determined by the quadratic photon-flux density dependence at the focus of the beam. (TPLSM resolution is about the same, but localization is superior to, that for CLSM [27].) Thus, background light arising from out-of-focus fluorescence in EPM may be dramatically reduced relative to TPLSM.

- The linear photon-flux-density dependence of two-photon absorption in EPM renders the resolution and localization independent of laser-power fluctuations. In TPLSM, these fluctuations alter the size and falloff of the two-photon absorbing region, thereby rendering the resolution and localization time dependent [9]. The effects of misalignment errors are similarly reduced in EPM relative to TPLSM.
- The low light level required for EPM admits the possibility of utilizing cw rather than pulsed sources of light. For entangled-photon beams that are relatively monochromatic, this can result in a substantial reduction of the deleterious effects associated with the dispersive broadening of short pulses traversing optical components in TPLSM [9], as well as improved beam control. Even for nonmonochromatic and pulsed entangled-photon beams, dispersive broadening can sometimes be nonlocally cancelled by proper choice of optical materials (this is a result of anticorrelations in the frequency components of the entangled photons, engendered by energy conservation [33,34]). One example in which the photons remain coincident occurs when twin beams travel through materials whose dispersion coefficients are equal in magnitude and opposite in sign. EPM can therefore accommodate the use of optical fibers for the transport and processing of light. In TPLSM, in contrast, femtosecond-duration optical pulses are substantially broadened and thereby reduced in magnitude by the dispersion associated with transmission through the microscope's optical components, which results in a decrease of the two-photon absorption probability [9]. The TPLSM optical pulse duration must therefore be chosen to be sufficiently long to avoid this effect.
- Because optical fibers can be used to transport light in EPM, there is the possibility of carrying out near-field entangled-photon microscopy (NFEPM).
- Materials with narrow two-photon absorption spectra can be effectively investigated with EPM because the frequency spectrum of the summed energy of the entangled photon pair is narrow [21,32]. This, again, results from anticorrelations in the frequency components of the entangled photon pair. In TPLSM, the material's absorption spectrum must be sufficiently broad to accommodate the spread in the summed energy of the two photons, demanding that the optical pulses not be too short.

As with TPLSM, the emitted fluorescence radiation in EPM need not be focused, descanned, or passed through special optics, thereby permitting detection to be carried out in a greater variety of configurations than with CLSM. This can be beneficial for imaging in certain media, such as those that scatter light more strongly at shorter wavelengths than at longer wavelengths. Longer wavelength excitation light can then penetrate into the interior of a sample while the shorter wavelength emitted fluorescence can be detected over a relatively large solid angle. Another feature shared by EPM and TPLSM is the ability to use markers other than emitted optical fluorescence photons to signal the presence of two-photon excitation. In analogy with two-photon scanning photochemical microscopy (TPSPM) [27], entangled-photon absorption can cause a photoactivatable probe to release neurotransmitter molecules; the binding of the free neurotransmitter to nearby ligand-gated ion channels can then be detected via the cellular ionic current flow. The entangled version might be called entangled-photon photochemical microscopy (EPPM). Other implementations can also be envisioned; examples

include the localized release of chemicals, electrons [11], or thermal/acoustic emissions engendered by the absorption of entangled-photon pairs.

Finally, entangled-photon microscopy can be extended to third order by using a scheme in which three entangled photons conspire in the absorption, in analogy with three-photon laser scanning fluorescence microscopy [35-37]. Further extension to yet higher-order implementations that make use of multiply entangled photons, can also be envisioned.

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