Light-sheet engineering using the Field Synthesis theorem

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Abstract
Recent advances in light-sheet microscopy have enabled sensitive imaging with high spatiotemporal resolution. However, the creation of thin light-sheets for high axial resolution is challenging, as the thickness of the sheet, field of view and confinement of the excitation need to be carefully balanced. Some of the thinnest light-sheets created so far have found limited practical use as they excite too much out-of-focus fluorescence. In contrast, the most commonly used light-sheet for subcellular imaging, the square lattice, has excellent excitation confinement at the cost of lower axial resolving power. Here we leverage the recently discovered Field Synthesis theorem to create light-sheets where thickness and illumination confinement can be continuously tuned. Explicitly, we scan a line beam across a portion of an annulus mask on the back focal plane of the illumination objective, which we call C-light-sheets. We experimentally characterize these light-sheets and demonstrate their application on biological samples.

Introduction
Light-sheet fluorescence microscopy (LSFM) has been transformative for volumetric imaging of single cells up to entire organisms, as it minimizes sample irradiation and allows efficient and rapid 3D imaging [1–3]. LSFM provides excellent spatiotemporal resolution, but is commonly not considered a super-resolution technique, as its spatial resolving power is still diffraction limited. Nevertheless, some LSFM implementations have been developed that can attain 300 nm scale axial resolution [3–7], effectively doubling the resolution of confocal microscopy, the workhorse in 3D microscopy. This is enabled by the larger set of angles that the separate illumination and detection objectives cover compared to a single objective microscope system. As such LSFM can improve the z-resolution over epi-fluorescence microscopes without compromising temporal resolution or requiring specialized fluorophores or nonlinear optical phenomena.

A driver for z-resolution in light-sheet microscopy is the thickness of the sheet, which in turn is governed by the laws of diffraction. For Gaussian beams, the thickness of the sheet is coupled via beam divergence to the confocal parameter of the beam waist, which dictates over what propagation distance a light-sheet can be approximated. While in principle Gaussian light-sheets with sub-micron thickness can be created, their confocal parameter is usually shorter than a typical cell, which makes them ill-suited for practical imaging.

Propagation invariant beams, such as Bessel [8] and Airy [9, 10] beams, and optical lattices, can in principle overcome the divergence of Gaussian beams. To create a light-sheet, such a beam is rapidly scanned laterally, a process known as digitally scanned light-sheet (DSLM) [2]. However, when propagation invariant beams are used for DSM, for a given light-sheet thickness, any increase in confocal parameter is paid by reduced confinement of the excitation energy into the sheet. This has been first discovered with Bessel beam light-sheets [11–14]: while the main lobe of such a light-sheet is very narrow, it is accompanied by a large beam skirt that may contain over 90% of the energy within the light-sheet [6]. Thus large amounts of out-of-focus light are excited, which generate unwanted out-of-focus fluorescence and lead to accelerated photo-bleaching.
To address this problem, the Betzig lab introduced lattice light-sheet microscopy (LLSM) [3], which generates a light-sheet by coherent superposition of many Bessel beams. By carefully adjusting the spacing between the individual beams, optical lattices can be generated that can balance the thickness and the confinement of the light-sheet. The most widely used sheet in LLSM is the square lattice, which offers excellent confinement, but consists of a much thicker main lobe than a Bessel beam light-sheet. For higher axial resolution, the hexagonal lattice was proposed, which has a thinner main lobe than the square lattice, however at the cost of much stronger sidelobes. So far hexagonal lattice light-sheets have been rarely used, presumably because the sidelobes are difficult to remove numerically.

Recently, we have discovered a new mathematical theorem called Field Synthesis that can be leveraged to create light-sheets in a more flexible and general way [15]. In Field Synthesis, a line is scanned over a mask conjugate to the back pupil of the illumination objective and a light-sheet is generated as an incoherent summation of the resulting instantaneous intensity distributions (see also figure 1). While we have shown previously that this method can be used to recreate existing light-sheets faithfully, we explore here the potential of Field Synthesis to generate new light-sheets that are tailored to specific applications. In particular, our goal was to create light-sheets that have properties in between hexagonal and square lattices. Here we present experimental results of these new light-sheets that we have termed C-light-sheets, which are characterized in transmission and with fluorescent nanospheres, and are applied to biological imaging.

**Concept: light-sheet design**

For the studies here, we restricted ourselves to an annular shaped pupil mask. The design rationales are outlined for light-sheet generation using the Field Synthesis approach (figure 1), where a line is scanned over a pupil filter in Fourier space. As shown in figure 2(a), when analyzing the image formation process for one particular scan position of this line, the Fourier components of the light-sheet are given by the autocorrelation of the pupil function, in this case resulting in three line segments. These components give rise in real space to intensity distributions as shown in figure 1(b), a sinusoidal pattern with a compact support. We note that a purely sinusoidal pattern would only result for an infinitely thin annulus mask. Such illumination pattern have recently been described as Bessel beams [16, 17], however, mathematically they are Cosine patterns bound by an envelope. For simplicity, we will call such a beam a Cosine-Gauss beam.

Using such a Cosine-Gauss beam as a light-sheet, we can compute the overall LSFM optical transfer function (OTF), which is the convolution of the Fourier components of the illumination and the wide-field OTF (figure 2(b)). As it can be seen, there are gaps between the main three lobes of the LSFM OTF. This has been...
previously described in standing wave microscopy [18], where a sinusoidal illumination pattern is created by interfering two laser beams from two opposing objectives. A remedy was readily found by Lanni and later Gustafsson by using multiple standing waves of varying axial frequencies to fill the gaps [19, 20].

This can be done for light-sheet illumination as well. If we scan a line over the full annulus (figure 2(c)), a continuum of Cosine-Gauss beams is superimposed that contain the lowest spatial frequency (edges of the annulus) up to the highest spatial frequency that the optical system can produce (center of the annulus). As we have previously shown, the resulting time-averaged intensity distribution corresponds to a Bessel beam light-sheet [15] (figure 2(d)).

If we instead use Field Synthesis to create a square lattice light-sheet, the beam is stepped to three discrete positions in the pupil, two at the edges of the annulus and one at its center (figure 2(c)). The resulting light-sheet has significantly reduced ringing, but its main lobe is thicker than a Bessel beam light-sheet.

For a hexagonal lattice the two main orders are shifted more inwards to the center of the annulus, and hence create a light-sheet with higher spatial frequency content. Through simulations and experiments we have found that the central order is much weaker and does not contribute significantly to the final light-sheet (supplementary note is available online at stacks.iop.org/JPHOTON/2/014001/mmedia). Thus, in an approximation, one can describe the light-sheet as a single Cosine-Gauss beam. Such a light-sheet features a thin main lobe, at the cost of stronger sidelobes compared to a square lattice sheet (figure 2(e)). Imaging with strong sidelobes has been shown to be a challenge in other imaging modalities, such as 4pi [21] and ISM [20], and the removal of the resulting ghost images is difficult [22]. As a practical rule of thumb, the sidelobe strength should be kept below 50% (for a theoretical derivation, see Nagorni and Hell [23]). This is the main reason that 4pi
microscopy has only been widely used with two-photon excitation, which suppresses the sidelobes to a tolerable level [24].

We hypothesized that we could create light-sheets with advantageous properties if we superimposed the spatial frequency range that exists between a square and a hexagonal lattice light-sheet. A continuum of intermediate frequencies can be generated if we scan a line from the position of the main order of the square lattice to the corresponding position of the hexagonal lattice (see figure 2(c)). We expected that this would lead to a reduction in sidelobe strength (figure 2(e)) and also would fill gaps in the OTF. Since the scanning pattern in the pupil looks like a letter C, we chose to call them C-light-sheets. We note that there is some similarity of this approach to light-sheets using Mathieu beams [25, 26], which were also called truncated Bessel beams. However in our case, the light-sheets are not generated by scanning a confined beam laterally, but by summing multiple Cosine-Gauss beams, resulting in a higher spatial duty cycle that reduces photo-bleaching [15]. Further, our scanning approach is more flexible, as the scan range can be chosen to optimize thickness and out-of-focus blur, whereas the sectioned Bessel beams are generated by a fixed mask. Lastly, in our C-light-sheets, the laser scan does not start at the very edge of the annulus, but more inward, where the main order of the square lattice is located. Dispensing the lowest spatial frequencies makes the C-light-sheets a bit thinner.

For classifying the different light-sheets, we indicate the position of the laser line in normalized units of the annulus radius. As an example, the main order of the square lattice is located at a scan position of 0.89 of the normalized radius (figure 2(c)), while the hexagonal lattice main order is located more inwards, at 0.72 of the normalized radius. The C-light-sheet in figure 2(e) is scanned over a range of 0.72–0.89 of the normalized radius. To abbreviate these different light-sheets, we call in this example the square light-sheet ‘Squ89’, the hexagonal light-sheet ‘hex72’ and the C-light-sheet ‘C72–89’. The same abbreviation also applies to figures 3 and 4.

Methods

Optical setup
To create the light-sheets, we used the illumination train of our previously published Field Synthesis setup, but we used different objectives: we replaced the illumination lens with a Special Optics NA 0.69 lens and used a Nikon NA 1.1/25 × lens for detection. 3D intensity images of the light-sheets were acquired in transmission, as previously described [15]. The annulus of the pupil filter corresponded to an inner NA of 0.45 and an outer NA of 0.536. To create the C-light-sheets, we scanned the beam across only one half of the annulus (see also figure 3(a) for experimental results). Scanning both halves would not change the light-sheet itself but would add angular diversity that helps to suppress shadow artifacts. As of now, we have not implemented hardware control that would allow us to perform scanning on both sides of the annulus during one camera exposure.

Sample preparation
Bovine Type I collagen (Advanced Biomatrix, 5005-100) was labeled with AlexaFluor 488 NHS Ester (Fluoroprobes 1013-1) at 4°C overnight in sodium bicarbonate buffer and dialyzed overnight at 4°C in 0.2% acetic acid. Collagen samples were prepared by polymerizing 2 mg ml⁻¹ of non-fluorescently labeled collagen supplemented with AlexaFluor-488-labeled collagen in imaging holders. Briefly, 1 ml of non-fluorescent collagen solution was prepared by mixing 100 μl 10X phosphate buffered saline, 10 μl 1 N sodium hydroxide, 230 μl Milli-Q water and 660 μl of 3.0 mg ml⁻¹ collagen stock solution. Next, 25 μl of AlexaFluor488-labeled collagen was added to 475 μl of the non-labeled collagen to achieve 5% labeling density. Collagen was allowed to polymerize at 37°C in a humidified incubator, and stored in sterile phosphate buffered saline prior to imaging.

Native human retinal pigmented epithelium cells immortalized by HPV-16 (ATCC, CRL-2502) were used to generate stable cell lines overexpressing eGFP-Clathrin Light Chain (eGFP-CLCa, Loerke and Mettlen et al [27]) by retroviral infection. Cells were sorted for eGFP expression level (Moody Foundation Flow Cytometry, UTSW), and checked for incorporation of tagged CLCa. The cells used in these studies express 10 times more eGFP-CLCa than endogenous CLCa and exhibit a corresponding downregulation of endogenous to 10% as expected. eGFP-CLCa cells were cultured in DMEM/F12 supplemented with 10% FBS at 37°C in a 5% CO₂ atmosphere.

Deconvolution
For each light-sheet 200 nm fluorescent nanospheres (Polysciences, 17151-10) were imaged and an isolated bead with a high signal to noise ratio was selected as a PSF, which was then rotationally averaged. The rotationally averaged PSF was subsequently refined by a blind deconvolution routine in MATLAB 2019a (deconvblind) over ten iterations. This helped to remove some small artifacts introduced by rotational averaging. We then used the retrieved PSF from the previous blind deconvolution to deconvolve image data (using again deconvblind). We
limited the number of iterations to ten to avoid clipping of dim features and over-deconvolution (i.e. excessive deconvolution can produce overly optimistic resolution on point objects like beads).

**Results**

We acquired the 3D intensity distribution of different light-sheets in transmission and acquired point spread functions using 200 nm fluorescent nanospheres. In figures 3(a)–(c), a comparison of different light-sheets is given. In the first column, the intensity as measured in a conjugate pupil plane is shown. The second column from the left (figure 3(b)) shows the different light-sheets as measured in transmission. Table 1 gives values for the beam waist and propagation length of the light-sheets.

For a Bessel beam light-sheet (figure 3(a), first row), a line is scanned over the full annulus. If we look at the light-sheet and its profile (figure 3(c)), a pronounced beam skirt is visible. This is reflected in the corresponding
experimental point-spread function, which has a sharp peak at the center, but then gradually fades away. If we look at the corresponding OTF, one can see that the support is elongated in the axial direction, however the strength further away from the center is weak. This is a consequence of the strong excitation of out-of-focus light, which strengthens the lower frequencies in the LSFM OTF that correspond to the wide-field OTF.

In the next two rows, two hexagonal lattices are compared. Depending on the position of the line, the light-sheet exhibits sidelobes of varying strength (50% of the main lobe or higher). In the corresponding PSF, one can see that the main axial lobe can be squeezed, however, additional out-of-focus light is excited by the sidelobes. The corresponding OTFs feature two gaps in their support. This is a consequence of the strong sidelobes.

If we look at the square lattice light-sheet in the fourth row, it is obvious that it has very minimal sidelobes and thus excellent illumination confinement, however its PSF is much more elongated in the axial direction than in the case of the hexagonal lattices. Also, the OTF is very elliptical, meaning it has limited support in the axial direction.

In the last three rows, three different C-light-sheets are shown. In the corresponding axial profiles, one can see that the strength of the sidelobes is kept below 50% and that the intensity decays gradually without much ringing in the axial direction. The corresponding PSFs are more compact than the one of the square lattice, however they exhibit less out-of-focus blur than the hexagonal lattices. The OTFs have no gaps and show a more solid support compared to the OTF of the Bessel beam.

Table 2 lists the lateral and axial full width half maxima (FWHM) of beads before and after deconvolution. In this measurement, 100 nm fluorescent nanospheres (Invitrogen, F8803) were used. The PSF of the Bessel light-sheets did not deconvolve well, and the axial resolution remains poor with ~800 nm. In contrast, the square lattice light-sheet and the moderate C72–89 sheet showed little remaining sidelobes and improve the axial resolution to 577 and 478 nm, respectively. Interestingly, the two C-sheets with a larger scan range (C48–90 and C55–90) did not improve axial resolution any further than the less aggressive C72–89.

Table 1. Light-sheet properties as measured in transmission. Mean and standard deviation are listed.

| Light-sheet type | Axial FWHM (μm) | Propagation FWHM (μm) | # of average |
|-----------------|----------------|-----------------------|-------------|
| Bessel          | 0.37 ± 0.003  | 15.11 ± 0.076         | 6           |
| Hexagonal 72    | 0.30 ± 0.001  | 13.69 ± 0.215         | 6           |
| Hexagonal 79    | 0.39 ± 0.002  | 13.80 ± 0.174         | 6           |
| Square 89       | 0.69 ± 0.010  | 22.19 ± 0.607         | 6           |
| Cshape 48–90    | 0.40 ± 0.003  | 14.67 ± 0.057         | 6           |
| Cshape 55–90    | 0.42 ± 0.003  | 14.72 ± 0.098         | 6           |
| Cshape 72–89    | 0.51 ± 0.004  | 14.86 ± 0.073         | 6           |
We demonstrate methods such as LLSM. In fact, of the two lattice light-sheet options continuously tuned. This affords for much sheets. The width of the main lobe of the light-sheet and the amount of out-of-focus excitation can be achieved by scanning a beam only partially over an annular pupil.

Table 2. Lateral and axial resolution before and after deconvolution. Measured on 100 nm fluorescent nanospheres, averaged over 10 spheres for each light-sheet. Mean and standard deviation are listed.

| Light-sheet type | Before deconvolution | After deconvolution |
|-----------------|----------------------|---------------------|
| Light-sheet type | Lateral FWHM (nm)    | Axial FWHM (nm)     |
|                 |                      |                     |
| Bessel          | 295 ± 14.2           | 848 ± 45.5          |
| Hexagonal 72    | 319 ± 22.0           | 434 ± 24.5          |
| Hexagonal 79    | 313 ± 22.4           | 532 ± 21.8          |
| Square 89       | 300 ± 8.4            | 778 ± 18.0          |
| Cshape 48–90    | 303 ± 14.4           | 660 ± 36.5          |
| Cshape 55–90    | 304 ± 14.3           | 677 ± 35.9          |
| Cshape 72–89    | 305 ± 9.5            | 661 ± 14.7          |
|                 | 282 ± 11.2           | 818 ± 37.0          |
|                 | 312 ± 21.9           | 439 ± 26.2          |
|                 | 257 ± 16.7           | 421 ± 13.2          |
|                 | 219 ± 5.6            | 577 ± 17.4          |
|                 | 266 ± 11.7           | 550 ± 24.3          |
|                 | 238 ± 8.7            | 484 ± 22.2          |
|                 | 228 ± 6.8            | 478 ± 9.1           |

While the hexagonal lattices can improve the axial resolution further (429–431 nm), iterative deconvolution was not able to fully remove sidelobe structures, confirming the 50% sidelobe rule established for 4pi microscopy [23]. We hypothesize that the solid OTF support of the square and the C72–89 light-sheets help with deconvolution, especially in scenarios of low signal to noise ratios. Interestingly, this also held true for lateral resolution, where iterative deconvolution enabled a gain of about 1.36 for the square and the C72–89 light-sheets. For the other light-sheets, the gain in lateral resolution by deconvolution was notably smaller.

Next we volumetrically imaged collagen samples with Bessel, hexagonal and square lattice, and C72–89 light-sheets, followed by iterative deconvolution (figure 4). Figure 4(a) shows a cross-sectional view of collagen (maximum intensity projection) as imaged with the Bessel beam light-sheet. One can see that even after deconvolution, out-of-focus haze is still visible (inset on the left of figure 4(a)). In contrast, the square lattice light-sheet shows much higher optical sectioning and very little to no deconvolution artifacts (figure 4(b) and inset on the right). While the hexagonal lattice light-sheet has higher resolving power in the axial direction, iterative deconvolution cannot completely remove some of the sidelobes (figure 4(c) and inset on the left). In contrast, the C72–89 light-sheet enables artifact free deconvolution and resolves finer details than the square lattice light-sheet in the axial direction (figure 4(d) and inset on the right).

We further imaged ARPE cells labeled with eGFP clathrin light-chain. Figure 5(a) shows maximum intensity projections (MIPs) of ARPE cells as imaged with Bessel, hexagonal and square lattice, and three different C-light-sheets. Figure 5(b) shows the magnified regions (red box in figure 5(a), single slice and MIPs of some planes), which highlights that the Bessel and hexagonal light-sheets do not deconvolve properly without artifacts. In contrast, the square lattice and the C72–89 light-sheet resulted in very cleanly deconvolved data. The more aggressive C-light-sheets (C55–90 and C48–90) show in individual slices (figure 5(b), bottom) slight blur above and below the main lobe of its PSF. In a maximum intensity projection, these artifacts are less visible.

Discussion

We demonstrate flexible tuning of light-sheet thickness and confinement using Field Synthesis. This has been achieved by scanning a beam only partially over an annular pupil filter, a procedure that we have termed C-light-sheets. The width of the main lobe of the light-sheet and the amount of out-of-focus excitation can be continuously tuned. This affords for much finer control over these two parameters compared to previous methods such as LLSM. In fact, of the two lattice light-sheet options (square and hexagonal), we found that only the more conservative square lattice has a generally usable OTF. In contrast, the hexagonal lattice light-sheet exhibits gaps in its OTF, which make deconvolution much more demanding, and in our hands, results in artifacts in the reconstructed data. This was true for both blind deconvolution and Richardson Lucy deconvolution using experimentally measured PSFs.

Although we only demonstrated three C-light-sheets and two hexagonal light-sheets in this article, we have investigated more C-light-sheets and hexagonal light-sheets since it is very simple to generate different light-sheets with Field Synthesis. We have generated hexagonal lattice light-sheets with even stronger sidelobes (>50% of the main lobe) but they were not useful for practical imaging. We have also generated more aggressive C-light-sheets by scanning a larger portion of the annular pupil filter, which produced a stronger beam skirt, which caused challenges in the deconvolution step. Obviously, a C-light-sheet with a large scanning range converges to a Bessel beam light-sheet.

We think that our new light-sheets will enable light-sheet users more options than the current state of the art to adjust their axial resolution and excitation confinement to their imaging applications. We also think that the Field Synthesis theorem has given a more intuitive approach to understand the characteristics of popular light-sheets such as Bessel beams and optical lattices. Thereby Field Synthesis has allowed us to engineer new light-
sheets in a straightforward and rational way. In contrast, this would be difficult to realize in a lattice light-sheet microscope set-up, as the number of useful optical lattices is limited. It is thus our hope that more useful light-sheets may be discovered with Field Synthesis, potentially by using more complex pupil filters.

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