Image-inspired 3D multiphoton excited fabrication of extracellular matrix structures by modulated raster scanning

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Abstract: Multiphoton excited photochemistry is a powerful 3D fabrication tool that produces sub-micron feature sizes. Here we exploit the freeform nature of the process to create models of the extracellular matrix (ECM) of several tissues, where the design blueprint is derived directly from high resolution optical microscopy images (e.g. fluorescence and Second Harmonic Generation). To achieve this goal, we implemented a new form of instrument control, termed modulated raster scanning, where rapid laser shuttering (10 MHz) is used to directly map the greyscale image data to the resulting protein concentration in the fabricated scaffold. Fidelity in terms of area coverage and relative concentration relative to the image data is ~95%. We compare the results to an STL approach, and find the new scheme provides significantly improved performance. We suggest the method will enable a variety of cell-matrix studies in cancer biology and also provide insight into generating scaffolds for tissue engineering.

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References and links


1. Introduction

The native extracellular matrix (ECM) has intrinsic 3D complexity with size features over length scales of ~100 nm in diameter to several microns in length. In addition to providing structural support, the ECM directs cell shape, differentiation, migration, proliferation, as well as new tissue synthesis [1] by presenting a complex set of topographic, mechanical and biochemical cues to cells. It is now clear that in order to properly perform these functions cells recognize 3D spatial and biochemical domains of the ECM at the nano/microscale [2–5]. The regulation of these events has implications for proper functioning of normal tissues but also for dynamics in diseased states [6]. It is also becoming better appreciated that the ECM is altered in essentially all epithelial cancers and this remodeling continues through disease progression [7, 8]. Thus an improved understanding of the ECM in terms of structural organization and cell-matrix interactions could lead to the development of more efficacious treatments. For example, cell adhesion and migration become mis-regulated during ovarian and other cancers [9], and better insight into these processes could lead to targeted therapeutics. The ECM also influences differentiation of stem cells during development and ECM cues have been effective in initiating differentiation in vitro [10]. Creating biomimetic models of the ECM would afford such better investigations of the cancer biology and also provide insight into optimizing scaffolds for tissue regeneration/repair.

The optimal fabrication scheme needs to faithfully reproduce the nano/microstructured topography from the same multiple protein components that comprise the native structure. This goal of creating optimized biomimetic scaffolds has been an active area of research over the last decade, where many fabrication approaches including soft lithography, solid freeform methods, hydrogel synthesis, electrospinning, and chemical collagen synthesis have been investigated [11–14]. Our approach to this problem uses multiphoton excited (MPE) photochemistry, where analogous to multiphoton excitation fluorescence (MPEF) microscopy, the fabrication is confined to the focal volume, resulting in intrinsic freeform 3D capabilities [15]. In our implementation, proteins are solubilized in an aqueous environment in the presence of a photoactivator (e.g. Rose Bengal), which upon excitation and subsequent photochemistry results in covalent crosslinks between molecules. The resulting feature sizes are governed by the multiphoton point spread function at the laser excitation wavelength, and at high numerical aperture (NA) the resulting lateral and axial features are on the order of 300 and 700 nm, respectively [15]. We have developed purpose-built instrumentation for this work [16, 17] as well as new photoactivators for photocrosslinking [18, 19]. Additionally, through analysis of morphology and cytoskeletal architecture, we have shown how topography and ECM cues, together and separately, affect the morphology and migration of fibroblasts, ovarian cancer cells, and mesenchymal stem cells (MSCs) [20–22].

Much of the previous and current activity employing MPE fabrication has been in non-biological applications in polymeric systems [23, 24]. The research has been directed at making devices for several applications including 3D memory, photonic crystals, and miniature electronics [25–29]. By comparison, biological applications have been less explored (especially in terms of de novo fabrication with proteins), although they are on the increase. Several labs have used this approach to functionalize hydrogels with bioactive molecules, where these efforts have typically used two-photon lithography for surface and 3D modifications followed by chemical reactions to achieve the desired scaffold [14, 30–32].

Here we further exploit the power of MPE fabrication to create 3D models of the ECM by following a blueprint (both protein composition and morphology) based on high resolution microscopy data, e.g. two-photon exited fluorescence (TPEF) and Second Harmonic
Generation (SHG). We employ a new laser scanning approach that combines the attributes of raster and vector scanning, where the resulting protein concentration in the construct is reflective of the grey scale intensity in the image file. The results are compared to a previous method utilizing an STL/hatching approach [17], and the new method provides greater fidelity in reproducing the morphology and relative protein concentration in the image.

2. Methods

2.1 Materials

Fabrication solutions containing Bovine Serum Albumin (BSA, Sigma, St. Louis, MO) and Rose Bengal (Sigma) were prepared at 100 mg/mL and 1mM concentrations respectively. Fibronectin (FN; 1mg/mL, Millipore, Billerica, MA) was mixed with BSA and Rose Bengal at 1% v/v. A BSA monolayer adsorbed to the surface of silanized microscope slide serves as the non-specific background upon which the proteins are crosslinked.

2.2 Photochemistry

The two-photon excitation of the Rose Bengal photoactivator is induced by a 100 femtosecond titanium sapphire laser (Mira, Coherent, Santa Clara, CA) operating at 780 nm. The photochemistry proceeds through intersystem crossing to the long lived first triplet state and the generation of singlet oxygen which then attacks residues containing aromatic groups and free amines [33, 34]. The resulting radical protein then links to a second protein molecule, generating a covalent bond. A 20x, 0.75NA objective lens was used where the average power at the focus was kept constant (~100 mW at 78 MHz repetition rate).

2.3 Optical setup and instrument control

The purpose built multiphoton fabrication instrument has been described in detail previously [16] and the new salient features are shown in Fig. 1. The Ti:sapphire laser is coupled to an upright microscope stand (Axioskop 2, Zeiss, Thornwood, NY) and scanning is performed through a combination of laser scanning galvos (Cambridge Technologies, Bedford, MA) and a motorized stage (x-y-z, Ludl Electronic Product Ltd, Hawthorne, NY)) under LabVIEW control with a field programmable gate array (FPGA) board (Virtex-II PCI-7831R, National Instruments, Austin, TX) functioning as a DAQ [16]. The laser power entering the optical train is controlled through a 10 KHz electro-optic modulator (EOM, Conoptics, Danbuty, CT) and the laser is rapidly shuttered by a second, higher speed EOM (maximum 100 MHz, Conoptics). This instrument affords much greater flexibility in terms of scaffold size and complexity than could be achieved with a commercial laser scanning microscope. Parameters such as power, scanning area, the scan rate of galvos, the repetition of scanning pattern (#scans/layer) are set within the graphical user interface (GUI). The TPEF from entrapped residual photoactivator serves as the online fabrication diagnostic of crosslinking and is also read by the FPGA. The microscope is equipped with phase contrast and two-photon fluorescence imaging capabilities for characterization (e.g. immunofluorescence and quality control). We have shown previously that the minimum feature sizes for crosslinked protein structures correspond to the two-photon excited point spread function (PSF). For example, using 0.75 NA and 780 nm two-photon excitation, the lateral and axial “resolution” is about 600 nm and 1.8 microns, respectively [16]. Sub-resolution features have been obtained using MPE polymerization of polymers due to a chemical nonlinearity in the free radical kinetics [23]. However, this is not operative in protein crosslinking.
An FPGA was incorporated in the fabrication system to exploit parallelism of command executions (80 MHz clock rate) and to avoid bottlenecks in communications between the CPU and hardware through four of the First-In First-Out (FIFO) channels. The first two FIFO channels relay information from the main LabVIEW program to the FPGA to control the galvo mirrors and fast EOM shutter, while the other two record information from the PMT to create a live image of the fabrication making the communication between CPU and hardware near real-time. The source code of the instrument control software is freely available at: http://campagnola.molbio.wisc.edu/.

2.4 Modulated raster scanning

Our previous STL approach relied on providing x,y voltages to the galvo mirrors that were pre-calculated to match the desired pattern by manipulating the step size and speed. This was achieved through hatching the pattern (often manually) in CAD software (e.g. AutoCAD) to define the extent of polymerization or crosslinking. While successful in achieving freeform fabrication of 3D structures, this approach has both engineering and practical drawbacks. For example, the degree of polymerization was defined by the hatching density, where increased pixel dwell times were used to attain increased concentration and in the other limit, rapid steps were used for regions where no crosslinking was desired. This is not a highly accurate approach due to the inertia considerations of the galvos. Also, this approach is not optimal for fabricating scaffolds with rapidly spatially varying morphology, as it is not practical to rapidly vary the hatching density (e.g. within a few microns) to realize large changes in concentration (e.g. 5 fold).

To improve upon these limitations and better exploit the freeform capabilities of MPE fabrication, we implemented a new scanning approach we term modulated raster scanning that combines the strengths of raster and vector scanning. Here the galvos are raster scanned at their maximum speed (~40 KHz) while the laser is shuttered at much higher speed (10 MHz-40KHz) with the fast EOM. This is a more accurate approach than modulating the scanning speed and/or step size of the galvos, as they now run at a constant speed over the whole field of view with the same pulse energy and repetition rate. The average power entering the fast shuttering EOM is first set by the slower EOM, and this allows constant peak powers to be used during the scan process. As a result, the fractional “on-time” within each pixel defines the integrated exposure dose, which is a linear process. This then directly
correlates to the resulting protein concentration, i.e. we achieve is a linear map between the intensity in the original image data and fabricated structure. For example, if the galvos are run at the maximum scan rate of 40 KHz, this corresponds to a pixel dwell time of 25 μs. To calculate the desired dwell time, we divide the fully open 25 μs by the gray scale level (0-8 bits) in the original image. Therefore the shuttering speeds vary from ~10 MHz (fully closed) to 40 KHz fully open. We stress that there is no pulse picking utilized, and the repetition rate from the laser is still 78 MHz. This is an important capability, as using simple scaffolds (e.g. uniform matrixes), we showed that increased integrated laser exposure linearly resulted in increased crosslink density until all available reactive sites had been used [35, 36].

2.5 Image inspired fabrication

Figure 2 shows the flow chart of the steps used in fabrication process to generate 3D structures using modulated raster scanning. We begin with 3D 8-bit image files (.bmp or .tif) that were acquired with proper sampling with respect to the Nyquist criterion. This is crucial for accurate representation of the tissue as well as providing optimal 3D structural integrity of the corresponding fabricated scaffold. This is also important so that the resolution (i.e. feature sizes) in the fabricated construct properly matches that of the original image. 12 bit images can also be down-converted to 8 bit for this purpose. Then we the images are processed either for the modulate raster scanning approach or using the previously described STL model and scanning approach [17], which we use below for comparison (see Fig. 4).

[Diagram of the fabrication process]

The images can then further processed (e.g. with ImageJ) to generate patterns to optimally produce the desired structure, as this is not always possible due to overlapping fibers in the ECM that make it difficult to discern clear, discrete patterns. This processing includes combinations of filters (e.g. background subtraction, noise removal, despeckle, threshold, etc.) and enhancement (e.g. edge detection, Gaussian Blur and determination of the Eigenvalues of the Hessian matrix to uniquely identify fibers) [37]. As an example, Fig. 3 shows an original SHG image (single optical section) from a human ovarian malignancy as well as the same image processed with a binary threshold and also that resulting from the calculated Eigenvectors of the Hessian matrix. The final image patterns are then converted to 8-bit .bmp files and fed to the LabVIEW program for fabrication. The program also generates an MPE fluorescence image from the residual fluorescence of the photomultiplier to give an assessment of the fabrication quality of the pattern. The structure is then created one section
at a time based on the same respective optical section in the 3D image stack, with the same step sizes as used in the properly sampled image acquisition. The two-photon image is then acquired with the same objective and field size. By contrast, the STL method requires creating a 3D model from the original image data, then creating single sections for hatching, and features can be lost in the translation between these formats.

![Image](image-url)

**Fig. 3.** Example of image processing used in scaffold design. Far left is the original SHG image of the collagen in the stroma from an ovarian tissue malignancy. The middle image results from a threshold to remove the background noise. The far right image is obtained by calculating the Eigenvectors of the Hessian matrix of the SHG image to better accentuate fiber structures. Scale bar = 40 microns.

### 3. Results

We first demonstrate the capabilities of the modulated raster scanning approach by recreating a single section of a developing mouse heart (postnatal day 2). Figure 4(a) shows the original image of the mouse heart immunostained for collagen IV. This pattern was directly fed to the fabrication microscope without further processing to generate the structure shown in Fig. 4(b). For demonstration purposes, the protein solution was a mixture of BSA with 1% FN, as we have shown previously it is more efficient to include BSA for increased structural support as it has much greater solubility than matrix proteins (e.g. 50 vs. 1 mg/mL). Moreover, due to pH2 considerations FN is easier to work with than Col IV, although we have developed different photochemistries for the latter [19]. The two color overlap of the original image (green) and that of the fabricated construct (purple) is shown in Fig. 4(c). Here the white indicates a high degree of spatial overlap and few green regions are seen, indicating that few topographic regions were not reproduced. As a quantitative comparison, we can calculate the fidelity, which we define both in terms of spatial overlap between the model of the image data and the fabricated construct and also by the match between the respective gray scale intensities. This is performed by pixel by pixel co-localization tests using Fiji (ImageJ). This analysis showed that this structure matches the original image with 96% fidelity both in spatial localization and intensity. Thus the fabricated structure preserved most of the microarchitecture of the original image with the resulting relative protein concentration corresponding to relative intensities of the original pattern. To show the comparison between the new approach and the STL-hatching method, the raw image data was converted to an STL model and hatched in AutoCad (Autodesk, San Rafael, CA) to define the galvo step size and fabricated, and the two-color overlap with the image data is shown in Fig. 4(d). Of note are the green regions, showing that this fabricated structure lacks more of the features present in the original image than that produced by modulated raster scanning. Moreover, the hatching approach is essentially binary and the resulting scaffold contains uniform protein concentration across the field of view. Using the same co-localization tests as in Fig. 4(c), the STL generated structure had fidelity to original pattern of approximately 75%, and was much less than that using the modulated raster scanning approach.
We have also used modulated raster scanning to fabricate a 3D scaffold from a mixture of BSA and FN where the design was derived directly from a 3D confocal immunofluorescence (FN) image stack of the left ventricle of mouse postnatal day 2. The image stack, (taken near and around a blood vessel), was comprised of 22 optical sections, taken 1 micron apart and the rendering is shown in Fig. 5(a). Here we applied a threshold to the image data in preparation of fabrication, which used the same number of slices and volume (129 x129 x 21 microns) and the 3D rendering of the FN immunofluorescence in the fabricated structure is shown in Fig. 5(b). If we compare both pixel coverage and intensity between the thresholded image data and fabricated structure, fidelity of at least 95% was achieved. We note that some subtle changes in intensity and therefore structural features were lost when the threshold was applied.
Fig. 5. 3D renderings of confocal image (a) derived from FN in mouse left ventricle and resulting fabricated structure created through modulated raster scanning (b). The large feature indicated by the arrow is a blood vessel. The contrast was FN immunofluorescence in both images. Scale bar = 30 microns.

As another demonstration application for this technology, we show the feasibility of the fabrication technique to create 3D structures based on SHG image data of a human ovarian cancer. It has proven difficult to completely reproduce the original SHG images due to densely packed overlapping fibers of differing length. As an alternative, we utilized the Hessian eigenvalue approach (see Fig. 3(c)) to create a 3D model, as it captures the predominant fibers. The result of the 3D rendering of the raw data and fabricated construct (resulting from intrinsic fluorescence of entrapped Rose Bengal) are shown in Fig. 6(a) and 6(b), respectively. The structure was created at 0.75 NA, with an axial step size of 1 micron, which was the same as in the original image stack. The structure was comprised of the full 100 microns of thickness as in the original image stack however we were not able to image the entire axial extent due to a strong secondary filter effect of the entrapped Rose Bengal and instead show the top 50 microns. Good fidelity (~95%) is achieved, in terms of feature sizes (and area covered) and pattern intensity. The scanning electron micrograph (SEM) in Fig. 6(c) shows the fibrillar network in cross section throughout the 3D volume of the structure.

Fig. 6. 3D renderings of SHG image (a)) and fabricated structure (b) from a human ovarian cancer. Scale bar = 50 microns. (c) SEM of the fabricated structure taken at 600X magnification.

4. Discussion

We have demonstrated that the new modulated raster scanning approach provides high fidelity (~95%) in reproducing the morphology present in the ECM captured in high resolution optical microscopy images. This method is superior in reproducing spatially varying morphology than through the previously reported STL/hatching approach, which is essentially binary in terms of contrast. This is due to both inertia considerations related to
changing speed of the galvos to change the extent of polymerization but also due to the
hatching process used in creating the STL models. We note that modulated raster scanning
could be used in conjunction with STL models, although it this does not fully exploit the
power of the method given the binary nature of the models. Still, the STL approach has
utility. For example, if the image was not properly sampled in z, then 3D STL modeling is
necessary to interpolate missing data. The modulated scanning approach also has advantages
for this application over vector scanning. The latter is similar to random access, where the
galvos are only scanned where fabrication is needed, resulting in greater net scan speed [38].
Because of the intrinsic inertia, vector scanning is not appropriate for scanning over rapidly
spatially varying patterns (such as found in ECM morphology) and is best for fabricating
sparse features and/or similar features in close proximity.

Lastly we note it is also possible to rapidly switch the laser power and therefore change
the peak intensity to vary the extent of polymerization, however due to the optical
nonlinearity of two-photon absorption and possibility of photodamage, there is a narrow
range of peak power where crosslinking can be achieved [15]. Moreover, excessive power
can saturate the two-photon absorption transition, resulting in feature sizes larger than that
predicted by the two-photon excitation PSF. By contrast, varying the effective dwell time is a
linear process and greater dynamic range can be achieved, concurrent with less chance of
photodamage. Thus, maintaining constant peak power but rapidly shuttering the time of
exposure is the optimal approach for achieving the desired crosslink density.

While other nanofabrication technologies, e.g. photolithographies, can afford superior
resolution or minimum feature sizes, these methods do not have freeform capabilities nor can
produce crosslinked protein structures nor are compatible with aqueous environments. In
addition, it is difficult to create spatially varying features, and almost impossible to do so with
multiple components, as these techniques are designed to create replicates of the same
structure. While conventional 3D printing via stereolithography is a freeform method, the
minimum feature sizes are 50-100 microns, and the materials (e.g. plastics) have limited
biocompatibility. In sum, these well-established methods do not readily afford full
recapitulation of the complex native ECM microenvironment in a controlled and reproducible
manner and MPE fabrication with flexible instrument control can fill this missing gap in
technology.

5. Conclusions

We demonstrated a new instrument control approach for 3D multiphoton excited fabrication
we have termed modulated raster scanning. We demonstrated the utility of the method by
creating models derived directly from high resolution optical microscopy image data of ECM
structures. The new scheme outperforms previous fabrication methods both in terms of
flexibility in pattern generation and also in the resulting fidelity of the fabricated construct
relative to the image data. The approach will be a powerful method to create models of the
ECM to investigate cell-matrix interactions in diseased states (e.g. cancers) and also for
producing scaffolds for tissue regeneration/repair.

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