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Andreas C. Geiger, Casey J. Smith, Garth J. Simpson, "Multi-photon excited Fourier-transform fluorescence recovery after photobleaching (FT-FRAP) with patterned illumination," Proc. SPIE 11244, Multiphoton Microscopy in the Biomedical Sciences XX, 112442B (14 February 2020); doi: 10.1117/12.2545908

SPIE.

Event: SPIE BiOS, 2020, San Francisco, California, United States

Multi-photon excited Fourier-transform fluorescence recovery after photobleaching (FT-FRAP) with patterned illumination

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ABSTRACT

Fourier transform fluorescence recovery after photobleaching (FT-FRAP) is proposed and implemented for quantitatively evaluating diffusion and fractional recovery of proteins in complex matrices. Diffusion characterization of proteins is routinely performed for identification of aggregation and for interrogating molecular interactions with excipients. Conventional FRAP is noninvasive, has low sample volume requirements, and can support short measurement times by performing measurements over distances of only a few micrometers. However, conventional measurements are complicated by the need for precise knowledge of the bleach beam profile and potential errors due to sample inhomogeneity. In FT-FRAP, the time-dependent recovery in fluorescence due to diffusion is measured in the spatial Fourier domain, with substantial improvements in the signal-to-noise ratio, mathematical simplicity, representative sampling, and compatibility with multi-photon excitation. A custom nonlinear-optical beam-scanning microscope enabled patterned illumination for photobleaching a sample through two-photon excitation. The fluorescence recovery produced simple single-exponential decays in the spatial Fourier domain. Measurements in the spatial Fourier domain naturally remove bias from imprecise knowledge of the point spread function and reduce measurement variance from inhomogeneity within samples. Comparison between the fundamental FT frequency and higher harmonics has the potential to yield information about anomalous or spatially dependent diffusion with no increase in measurement time. Initial demonstrations of FT-FRAP using patterned illumination are presented, along with a critical discussion of the figures of merit and future developments.

Keywords: Fluorescence recovery after photobleaching (FRAP), spatial Fourier analysis, patterned illumination, multiphoton microscopy

1. INTRODUCTION

Fluorescence recovery after photobleaching (FRAP) is a widely accessible and ubiquitous method for interrogating diffusion.[1, 2] In FRAP, a region of fluorescently labeled sample is photobleached using short, high-intensity burst of light. After the bleach, unbleached fluorescent molecules diffuse into the bleached region and bleached molecules diffuse out of the region. This combined mobility results in a recovery of fluorescence intensity in the bleached region over time, the mathematical modeling of which enables recovery of diffusion information.

Despite the advantages of FRAP, quantitative analysis to recover accurate diffusion coefficients is typically complicated by the need for precise knowledge of the bleach pattern used for excitation. To reduce 1/f noise, FRAP is generally optimized using a small spot size for fast recovery, with a high bleach depth to maximize the signal-to-noise ratio (SNR). However, increasing the depth runs the risk of complicating reproducibility in the spatial profile of the bleach pattern by introducing nonlinearities from optical saturation and perturbations to diffusion from local heating.

Spatial Fourier analysis (SFA) is one of the more successful strategies used to date for addressing ambiguities in the point-spread function (PSF) for point-excitation.[3-6] In brief, diffusion in real space can be modeled by convolution of the bleach PSF with a time-varying Gaussian function. With a few notable exceptions for the PSF (e.g., Gaussian), this convolution generally produces a real-space recovery bleach pattern with no simple closed-form analytical solution. However, in the Fourier transform domain, the convolution corresponds to a simple multiplication. The decay curves for each spatial frequency in SFA can be used individually or collectively for recovering the diffusion coefficient. In this manner, the detailed functional form for the initial PSF become less critical in the analysis.[7] For point-excitation, SFA suffers somewhat by distributing the signal power from sharp features in the real-space image out over many low-amplitude frequencies in the SFA image, but the intrinsic signal to noise ratio can be recovered through simultaneous,

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Multiphoton Microscopy in the Biomedical Sciences XX, edited by Ammasi Periasamy, Peter T. C. So, Karsten König,
Proc. of SPIE Vol. 11244, 112442B · © 2020 SPIE · CCC code: 1605-7422/20/$21 · doi: 10.1117/12.2545908

collective analysis at multiple spatial frequencies. When one or a small number of frequencies are used, this distribution of power can result in a reduction in SNR, the cost of which represents a trade-off for the benefits in reducing ambiguities related to the PSF.

In this work, comb patterns for illumination during photobleaching were shown to support high SNR in subsequent Fourier analysis of the fluorescence recovery with multi-photon excitation. In brief, bleach patterns were selected to produce sharp features in the spatial Fourier transform, rather than sharp features in the real-space image, as shown in **Figure 1**. Patterned illumination distributed the power from the bleach over much larger regions in the field of view, removing many of the potential nonlinearities and ambiguities associated with highly localized excitation. Rapid line-scanning of the excitation beam enabled multi-photon excitation with negligible artifacts from local heating. Herein, FT-FRAP analysis is evaluated in proof of concept studies of model systems.

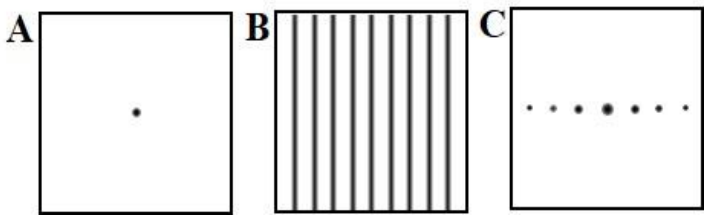

Figure 1. Schematic depicting A) conventional FRAP illumination, B) patterned illumination with comb excitation, and C) FT of patterned illumination. Conventional FRAP produces a sharp point in real space. FT-FRAP with patterned illumination produces sharp puncta in Fourier space.

2. EXPERIMENTAL METHODS

2.1 Two-photon excited fluorescence microscopy

The experimental apparatus for two-photon excitation in FT-FRAP is depicted in **Figure 2**. A tunable 80 MHz, Ti:sapphire, femtosecond laser (Spectra-Physics, Mai Tai) was used as the excitation source. The fundamental beam was raster-scanned across the sample using a resonant scanning mirror at 8.8 kHz (EOPC) for the fast-scan axis and a galvanometer mirror (Cambridge-Tech) for the slow-scan axis, both controlled by custom timing electronics built in-house.[8] A 10X, 0.3 NA objective (Nikon) was used to focus the beam onto the sample, and the two-photon excited fluorescence (TPEF) signal was collected in the epi direction through the same objective used for delivery of the excitation beam. The laser was tuned to 800 nm with a power of ~50 mW at the sample during imaging and ~500 mW at the sample during bleaching. A long-pass dichroic mirror (Chroma, 650DCXR) and a band-pass filter (Thorlabs, FGS900) were used to isolate the TPEF signal before it was detected by a photomultiplier tube (PMT) (Hamamatsu, H7422P-40 MOD). Responses of the PMT were digitized synchronously with the laser pulses by using a digital oscilloscope card (Alazar Tech, ATS9350) and mapped onto 512 × 512 images via custom software written in-house (MATLAB).[8] The TPEF videos were recorded at ~4 frames per second.

2.2 Comb bleach FT-FRAP

A simple change to the scan pattern of the galvanometer (slow axis) mirror was used to generate a comb bleach pattern at the sample. Following an initial period of low power for baseline TPEF microscopy of the full field of view, patterned bleaching was performed simply by changing the number of steps in the ramp function driving the galvanometer mirror from 512 (used for normal imaging) to an integer fraction of 512. The dwell time per step was also increased proportionally such that the repetition rate of the slow axis mirror was independent of the number of lines in the comb bleach pattern. As shown in **Figure 2**, a flip mount with a half-wave plate (HWP) was synchronized to switch the excitation source from low power to high power concurrently with the reduction in ramp steps. This protocol resulted in a comb bleach pattern as seen in **Figure 3A**. After ~2 seconds at high power the flip mount was removed from the beam path to reduce the laser power, and the number of steps for the slow axis mirror was changed back to 512 to facilitate normal imaging of the fluorescence recovery at low power.

2.3 Data analysis

Analysis of FT-FRAP data was performed using custom software written in-house (MATLAB). A 2-dimensional FT was taken of each image. FT-FRAP curves were recovered by integrating over peaks in the FT magnitude. A one-parameter fit was performed to recover the diffusion coefficient using **Equation (1)**, where t is time, $\bar{\nu}_x$ is spatial frequency, n is harmonic, $\tilde{C}$ is concentration in the spatial frequency domain, and D_{xx} is the diffusion coefficient.

$$\tilde{C}(\bar{\nu}_x, t) = \tilde{C}(n\bar{\nu}_x, 0)e^{-4\pi^2 (n\bar{\nu}_x^0)^2 D_{xx} t} \quad (1)$$

Uncertainties in the fits were calculated based on the curvature of χ^2-space in the vicinity of the minimum.

2.4 Sample preparation

A solution of 1 mg/mL FITC-bovine serum albumin (BSA) purchased from Invitrogen was used to evaluate the FT-FRAP approach. These fluorescently labelled molecules were solubilized in glyercol/water (2:1). Solutions were mixed thoroughly prior to FRAP analysis.

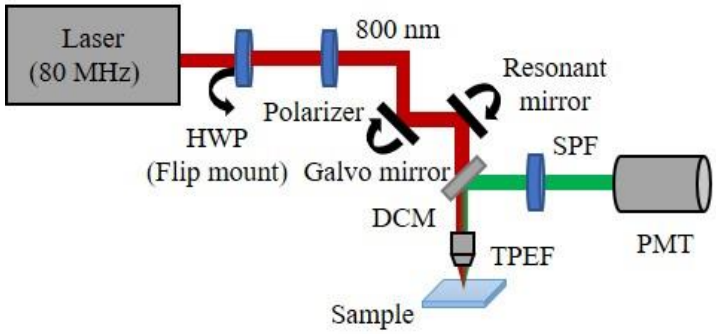

Figure 2. Instrument schematic of nonlinear-optical beam-scanning microscope built in-house for FT-FRAP. A half-wave plate (HWP) on a flip mount is used to modulate from low power (50 mW) to high power (500 mW) during the bleaching period. Beam scanning is performed with galvanometer (slow axis) and resonant (fast axis) mirrors. HWP = half-wave plate, DCM = dichroic mirror, SPF = short-pass filter, TPEF = two-photon excited fluorescence, PMT = photomultiplier tube.

3. RESULTS AND DISCUSSION

Following photobleaching, spatial Fourier transformation produced sharp puncta with symmetric amplitudes about the origin peak, as shown in **Figure 3** for a 1 mg/mL solution of FITC-BSA dissolved in a 2:1 solution of glycerol/water. **Figure 3A** shows an image of the sample immediately following a comb bleach. The 2-dimensional Fourier transform of this image is shown in **Figure 3B**. Note that the comb pattern is expressed as a series of multiple harmonics in the frequency domain. As diffusion progressed, the amplitude decayed with a time-dependent trend in excellent agreement with a simple exponential loss predicted by **Equation (1)**. **Figure 3C & 3D** show the exponential decay in the magnitude of the first and second harmonic peaks from the 2-dimensional Fourier transform. From **Equation (1)**, the exponential decay constant was directly related to both the diffusion coefficient and the periodicity of the sinusoidal bleach pattern. The FT-FRAP analysis clearly provides high signal to noise bleaching curves by combining the analysis over the entire field of view in the real-space image. Comb bleach patterns enabled simultaneous analysis over multiple length scales through the nth spatial harmonics, $\bar{\nu}_{x,n}$. Theoretical predictions in **Equation (1)** suggest expectation of exponential decay of each impulse with a decay constant given by $\tau = 1/\left[4\pi^2 \left(n\bar{\nu}_x^0\right)^2 D_{xx}\right]$. Consistent with this analysis, faster decays are expected for the higher harmonics, which correspond to shorter characteristic length scales. Because of the quadratic dependence, the fourth harmonic is expected to recover 16-times faster than the first harmonic for normal diffusion. Despite the notable differences in the decay times, the diffusion coefficients recovered by analysis of the decay curves in **Figure 3** were within experimental error of each other, consistent with theoretical predictions for normal diffusion.

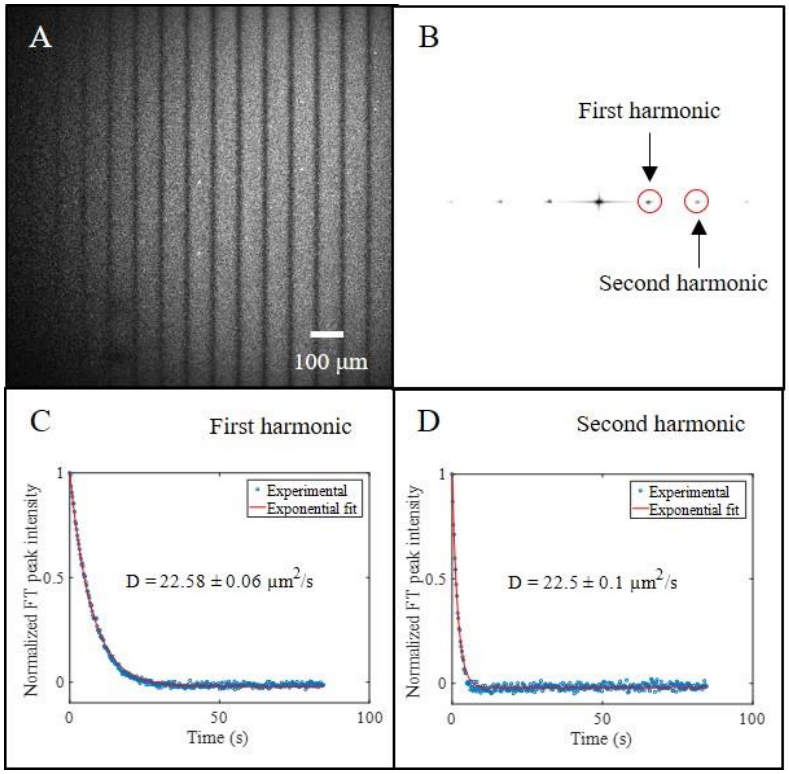

Figure 3. FT-FRAP with comb bleach of FITC-BSA dissolved in a 2:1 solution of glycerol/water. A) Image of the solution immediately subsequent to a 16-line comb bleach. B) 2D-FT of (A). C) Fluorescence recovery measured at the fundamental of the 2D-FT. D) Fluorescence recovery measured at the 2nd harmonic of the 2D-FT.

It is worthwhile to compare this FT-FRAP approach using patterned illumination with previous studies employing spatial Fourier analysis (SFA) of FRAP measurements. In those prior studies, Fourier analysis was performed to aid in interpretation of recoveries using conventional bleach illumination of localized points. SFA provided similar computational benefits in the mathematical simplicity arising in the Fourier domain. However, FT-FRAP has a major signal to noise advantage over conventional point-illumination. In the numerator, FT-FRAP supports major increases in the signal power by distributing the bleach amplitude over the entire field of view, whereas conventional point-illumination saturates at a much lower integrated signal power. In the denominator, patterned illumination enables shifting of the signal to a quiet spatial frequency for noise suppression. Analysis of natural images suggests a power spectrum obeying a $1/\bar{\nu}$ dependence.[9] For optical detection in the shot-noise limit, the variance in signal is proportional to the mean. Since visible photons are often detectable with signal to noise approaching the shot-noise limit in instrumentation optimized for FRAP, it stands to reason that the noise in the Fourier domain will also scale with the signal power in an image with natural contrast. Consequently, the noise power spectrum is also expected to scale with $1/\bar{\nu}$, in direct analogy with $1/f$ noise in electronics. As in electronics, shifting of the signal to a frequency regime with lower noise through modulation can provide a substantial noise reduction.

4. CONCLUSIONS

In this work, FT-FRAP with patterned illumination has been demonstrated experimentally. Relative to conventional point-bleach FRAP, FT-FRAP has the advantages of mathematical simplicity and higher SNR. Proof-of-concept measurements with a model system show good agreement with theory. Future fundamental work includes experiments to test the ability of FT-FRAP with patterned illumination to characterize anomalous diffusion and binding-unbinding behavior. FT-FRAP can also be implemented in a variety of application spaces including measurement of mobility in pharmaceutically relevant matrices, quantification of protein aggregation, and live-cell imaging.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge support from NSF-GOALI grant no. CHE-1710475.

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