Stain-Free Detection and Quantification of Bone Marrow Fibrosis Using Two-Photon Excitation and Second Harmonic Generation Microscopy

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Background

Bone marrow fibrosis is routinely assessed in the diagnostic work-up and prognostic evaluation of patients with known or suspected myeloproliferative neoplasm (MPN). Conventional evaluation of bone marrow fibrosis in cases of MPN is performed on reticulin- and trichrome-stained slides and is semi-quantitative and subjective. MPN grading systems, including the recently revised European consensus (EC) scoring system that uses a 0-3 scale, have been shown to have numerous limitations, including interobserver variability due to the subjective nature of scoring stained slides.

Two-photon excitation (2PE) signals are generated using a mode-locked pulsed laser beam to excite endogenous fluorophores. In 2PE, two photons of lower energy are simultaneously absorbed by a molecule (Figure 1). If the excited molecule is flourescent, it will emit a photon just as one would expect from the absorption of a single photon of greater energy. However, unlike one-photon confocal microscopy, 2PE uses near infrared photons which penetrate deeper into tissue with no absorption in out-of-focus areas (Figure 1). Excitation occurs only at the focal point of the laser where photon density is greatest, resulting in less risk of tissue damage. In addition, 2PE allows for stain-free quantification of unstained tissue structures as a result of intrinstic autofluorescence emissions.

Second harmonic generation (SHG) occurs nearly simultaneously with 2PE; two photons interacting with a nonlinear material effectively combine to form a new photon with twice the energy, and therefore twice the frequency and half the wavelength of the inital photon (Figure 2). A prerequisite for SHG is that the sample must exhibit a high degree of nonlinear molecular organization in order to generate appreciable SHG signals. As a result, SHG is highly specific for fibrillar collagens.

Because 2PE and SHG involve different contrast mechanisms they can be used in tandem to provide complimentary information regarding tissue structure and function (Figure 3). We investigated the applicability of two-photon excitation/second harmonic generation laser scanning microscopy (2PE/SHG) for quantification of fibrosis in unstained bone marrow core biopsy samples and compared its performance to the EC scoring system and a stereology-based quantitative method.

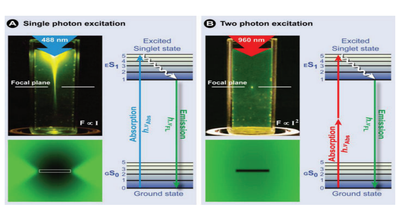


Figure 1. (A) One-photon excitation occurs through the absorption of a single photon. (B) 2PE occurs through the absorption of two lower-energy photons via short-lived intermediate states. In one-photon excitation, fluorescence excitation is observed throughout the path of the laser beam. In 2PE, excitation is restricted to a small volume in the focal plane of the sample where photon density is greatest. The result is no absorption in out-of-focus areas and less tissue damage. Image from Ishikawa-Ankerhold, et al., Molecules. 2012;17:4047-132.

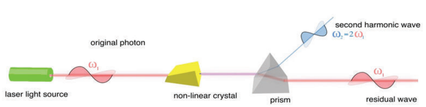


Figure 2. Second harmonic generation

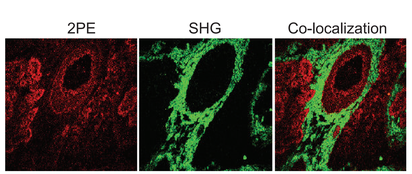


Figure 3. 2PE and SHG involve different contrast mechanisms and when used in tandem can provide complimentary information on structure and function in unstained tissue sections.

Materials and Methods

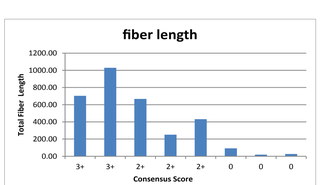
Bone marrow core biopsy samples submitted with an indication of MPN were selected or the study. An experienced hematopathologist reviewed the reticulin-stained slides and confirmed the EC score for 8 samples selected for study inclusion. The European consensus scores of the study samples included 0, 2+ and 3+ fibrosis.

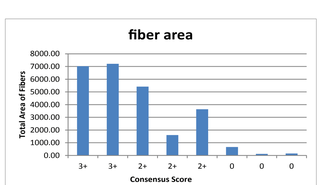
Unstained 4µm sections tissue sections underwent 2PE/SHG using the Genesis® 200 (Histoindex Pte, Ltd., Singapore). The Genesis® 200 utilizes an femtosecond erbium fiber laser and a 20x objective to acquire multi-tiled images (600µm^2 total area) from each sample, resulting in a spatial resolution of approximately 0.2µm. Image analysis was performed using proprietary software developed by Histoindex. Analysis parameters assessed included 2PE/SHG ratio, aggregated fiber percentage, total number, area, width, and length of fibers, and number of fiber cross-links.

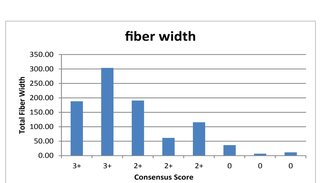
Following 2PE/SHG imaging, the tissue sections were stained with reticulin and scanned using the Aperio ScanScope (Leica Biosystems, Buffalo Grove, IL). Fiber length density (FLD) was calculated using an approach adapted from stereology. To calculate FLD, the number of reticulin-stained fibers that crossed over a fixed line distance of 239.6µm was manually counted (Figure 4).

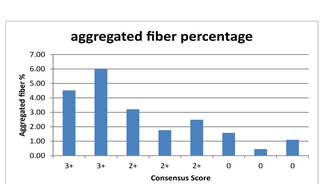
Results

2PE/SHG imaging segregated unstained bone marrow biopsy core samples with fibrosis from those without fibrosis. Binomial logistic regression showed a high degree of correlation between 2PE/SHG analysis and EC score for the majority of the parameters evaluated (Table 1). Total area of fibers (p = 0.004) and total length of fibers (p = 0.008) demonstrated the most significant degree of correlation. Interestingly, FLD using a stereology-based approach, also showed a significant correlation (p = 0.001) with the EC score. Cross-linking of fibers showed a trend but did not reach statistical significance (p=0.062).









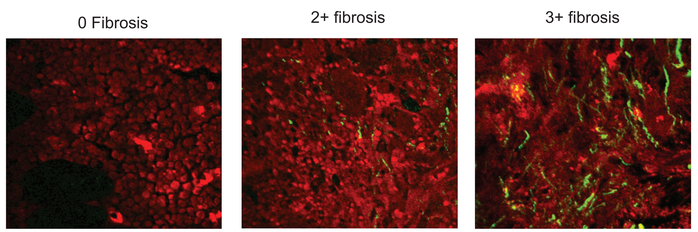


Table 1. Correlation between 2PE/SHG image analysis and European consensus score

2PE/SHG parameter	p-value
2PE/SHG ratio	0.054
aggregated fiber percentage	0.015
total number of fibers	0.023
total area of fibers	0.004
total width of fibers	0.016
total length of fibers	0.008
number of fiber cross-links	0.062

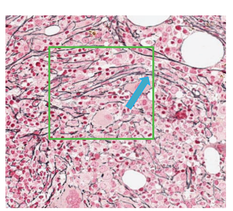


Figure 4. Fiber length density (FLD) is measured by manually counting the number of fibers that cross a fixed line distance (one arm of a fixed green square). The arrow points to a fiber that would be included in the FLD calculation.

Conclusion

2PE/SHG image analysis is a promising novel technique to be applied in the quantification of bone marrow fibrosis with performance equivalent to a stereology-based approach. This method produces images with the resolution of standard histology and eliminates the subjective scoring associated with grading trichrome- and reticulin-stained slides. Further studies with large sample size are needed to validate the utility of this method, however; several parameters can now be evaluated, as well as compared, to the biology of MPNs.

Disclosure Statement

There are no relevant conflicts of interest to disclose