Use of Second Harmonic Generation (SHG) and 2-Photon Emission (2PE) Imaging to Quantify and Describe the Structure of Tissue Fibrosis in the Rat TNBS Intestinal Fibrosis Model

University of Michigan Medical School

Johnson, Laura[1]; Chen, Li[3]; Rodansky, Eva S.[1]; Moons, David S.[2]; Petitjean, Mathieu[3]; Higgins, Peter D. R.[1]; Stidham, Ryan W.[1]

[1]Internal Medicine, University of Michigan, Ann Arbor, MI, United States. [2]Genesis Imaging Services, Pharmanest, Princeton, NJ, United States.
[3]Pathology, University of Michigan, Ann Arbor, MI, United States.

BACKGROUND

- Intestinal fibrosis is the dominant cause of complication and surgery in Crohn's disease.
- Strictures have increased collagen content, but collagen structure and networks in fibrotic intestine are poorly understood.
- Architectural changes in the collagen matrix induce and propagate fibrosis.
- However, with current methods, it is difficult to quantify collagen organization in tissue.
- Trichrome staining is the histological standard to identify collagen. However, it is not amenable to quantification (or automation)
- Second Harmonic Generation (SHG) imaging and two-photon excitation fluorescence (2PE) imaging enables:
  - Stain-free and label-free imaging
  - Preserves tissue microarchitecture
  - Can analyze standard histological sections (i.e. FFPE)
  - Extremely sensitive (0.39 μM resolution at 20x)
  - Quantification of tissue collagen content
  - Quantification of collagen fiber organization

AIMS

1) To determine whether SHG, 2PE can detect changes in tissue collagen associated with the progression of intestinal fibrosis

2) To determine if collagen fiber organization correlates with biochemical and histological fibrosis

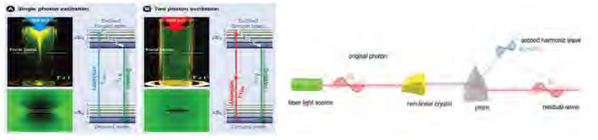
4) To determine whether SHG and 2PE can differentiate between normal, inflammed, and fibrotic intestine

METHODS

Rat TNBS colitis and fibrosis model

Group	TNBS cycles	Disease phenotype
PBS	0	normal
TNBS d2	1	inflammatory
TNBS d4	1	inflammatory
TNBS d7	1	inflammatory (healing)
TNBS d14	2	mixed inflammation/fibrosis
TNBS d28	4	fibrotic

- Female Lewis rats
- Intrarectal TNBS (2,4,6-Trinitrobenzenesulfonic acid)
  - acute (d2, d4, d7) single, 15 mg dose
  - chronic (d14, d28) weekly, escalating doses (15 mg to 60 mg)
- Outcomes: Fibrotic gene expression (col1A1, col III, IGF-1, TGFβ)
  Inflammatory gene expression (IL-1β, TNFα)
  αSMA protein expression
  Histology (Trichrome staining for collagen)

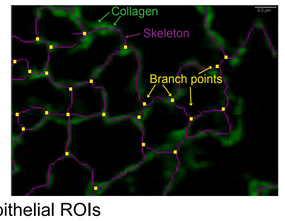


SHG and 2PE Imaging

- Unstained, deparaffinized FFPE slides
- GENESIS[R] 200 imaging system (HistoIndex), high (0.39 μM) resolution
- Two optical methods
  - SHG Second Harmonic Generation method (transmission), 390 nM (collagen I, III)
  - Two photon fluorescence (2PE), 550 nM (NADPH, NADH)
- Tissue ROIs delineating the epithelium, submucosa (lamina propria) and muscularis
- ROI selections were reviewed by a pathologist (D.M., blinded to the experimental groups)
- Image Analysis (proprietary algorithms/software) of collagen
  1. Collagen content
  2. Reticulation Index (%) = # of branching points/total length of the skeleton.
  3. Tortuosity = walking (geodesic) length/distance between the fiber end points.

Outcomes

- SHG collagen objects, tortuosity, and reticulation
- Histological comparison of SHG to Trichrome staining
- Gene expression
  Inflammatory genes: IL-1β, TNFα
  Fibrotic genes: col1A1, col III, TGFβ, IGF-1
- αSMA Protein expression

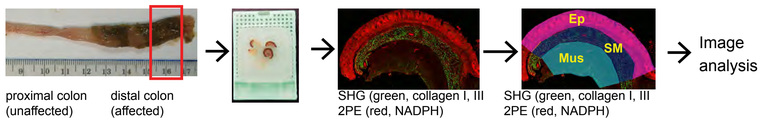


Analysis

- Analyzed full thickness, submucosa, muscularis and epithelial ROIs
- Rationale for tissue sub-analysis
  - Epithelial loss due to ulceration (acute phase) or scarring (fibrotic phase)
  - Fibrosis initially develops from activated lamina propria myofibroblasts
  - Muscularis hypertrophy and architectural distortion via collagen infiltration
- R package (Pearson correlation) cor.test

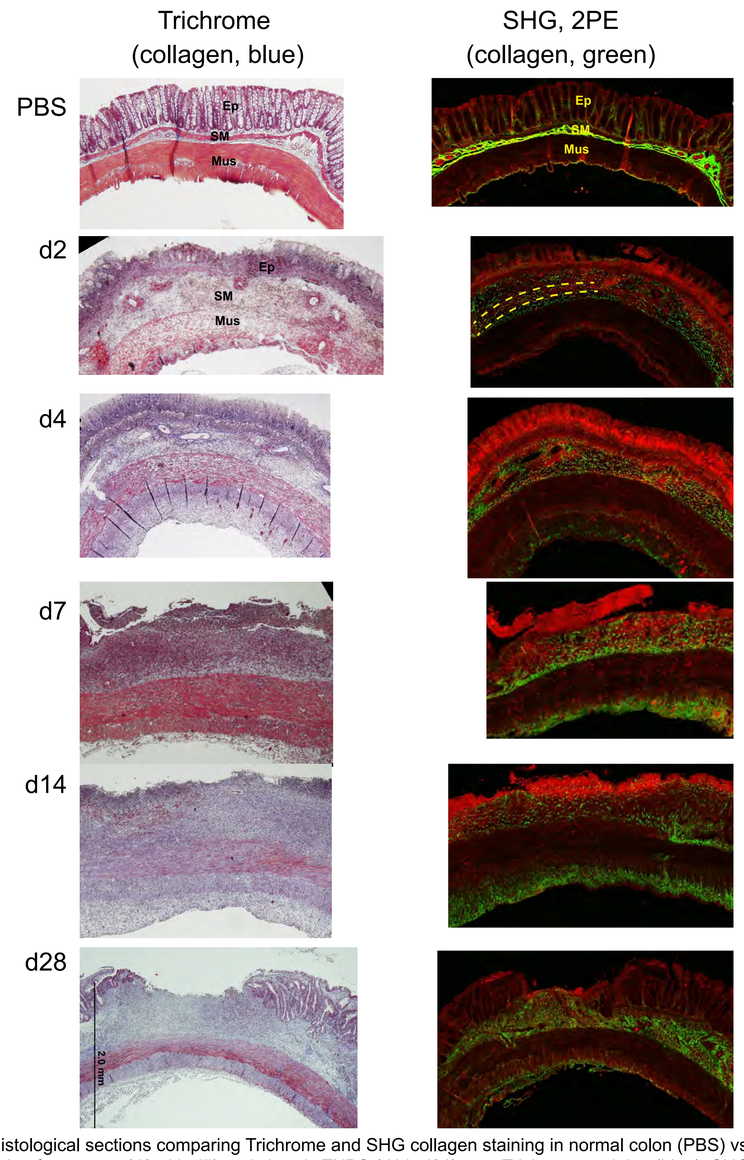
RESULTS

Figure 1. SHG, 2PE imaging and identification of regions of interest (ROI's) in rat distal colon.



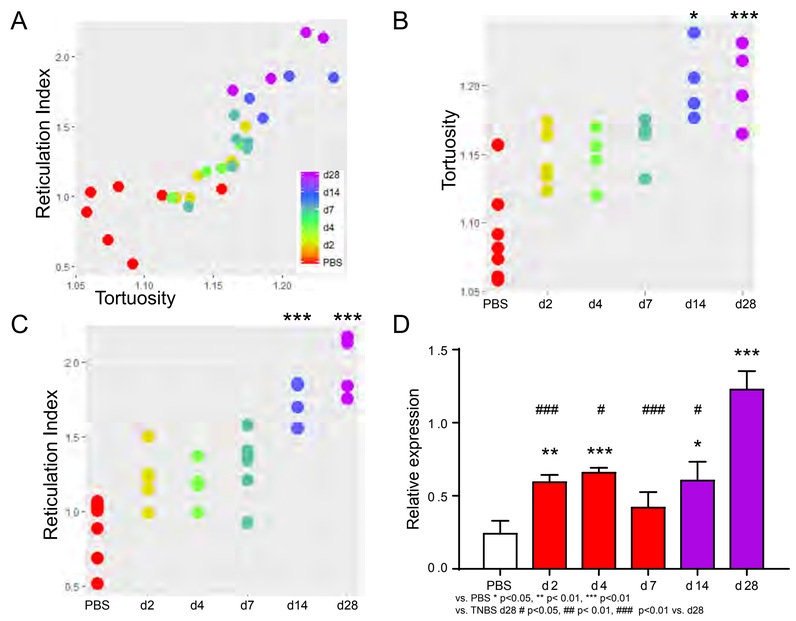
FFPE histological blocks are prepared from distal colon. Blocks are deparaffinized prior to SHG and 2PE imaging. 2PE images NAD/NADPH (red, autofluorescence). SHG detects non-centrosymmetric molecules collagen I, III (green). ROIs, from well-oriented, artifact-free sections were selected (Ep = epithelium, SM = submucosa/lamina propria, Mus = muscularis). A proprietary algorithm calculates total collagen objects and collagen organization (reticulation and tortuosity).

Figure 2. SHG collagen imaging corresponds to Trichrome collagen staining.



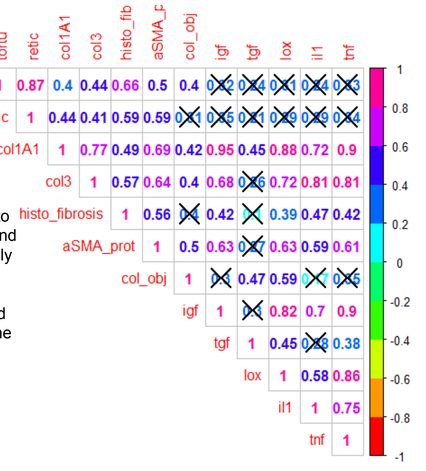
Histological sections comparing Trichrome and SHG collagen staining in normal colon (PBS) vs. colon from acute (d2, d4, d7) and chronic TNBS (d14, d21) rats. Trichrome staining (blue). SHG signal (green). In the normal colon, collagen is tightly organized within the submucosa/lamina propria (SM). In the acute colitis phase (d2, d4, d7), the SM collagen is dispersed by tissue edema. However, the collagen architecture is maintained as evidenced by concentric rings of collagen (yellow dashed line in d2). In the chronic (fibrotic) disease (d14, d28), disorganized collagen deposition distorts the entire architecture of the SM, and extends into the muscularis layer. An increased NAD/NADPH autofluorescence (red) in areas was observed in a majority of acute sections and a subset of the d14 sections. However, no increased signal was observed in the most fibrotic group (d28). High NADPH staining co-localized with neutrophils and with extra tissue neutrophil exudate, suggesting this may reflect increased oxidative stress.

Figure 3. Collagen structural changes (tortuosity and reticulation) precede myofibroblast activation in the submucosa.



(A) Scatter plot illustrating the direct relationship between SHG collagen reticulation and tortuosity in the submucosa from normal (PBS) vs. TNBS treated rat colon. (B) SHG tortuosity significantly increases at d14 and d28 (p = 0.02, p = 0.009) compared to d7. (C) Similarly, SHG collagen reticulation increased in the chronic (fibrotic) groups d14 and d28. Intestinal fibrosis is mediated by activated myofibroblasts (MFs) within the lamina propria/submucosa. (D) αSMA protein expression, a marker of tissue fibrosis and activated MFs, is significantly induced at d14 and d28 (2-fold, p = 0.013). In the muscularis, increased tortuosity is also observed at d14 and d28 (data not shown).

Figure 4. SHG tortuosity and reticulation correlate with biochemical markers of fibrosis, not inflammation.



Pearson correlation matrix comparing SHG tortuosity (tortu), reticulation (retic), and collagen objects (col_obj) to histological, fibrotic and inflammatory gene expression, and αSMA protein expression. Tortuosity and reticulation highly correlated with each other (0.87) and histological fibrosis scoring (0.66, 0.69). Moderate but statistically significant correlations were observed between SHG reticulation and tortuosity and αSMA protein expression and collagen gene expression (col1A1, col III). Neither tortuosity nor reticulation correlated with inflammatory markers (IL-1β, TNFα). Heatmap scale indicates correlation strength. Significant ($p < 0.05$) correlations are displayed.
X = $p > 0.05$

CONCLUSIONS

- SHG imaging reproduces Trichrome staining for collagen.
- SHG detects collagen I and III structural changes in fibrotic intestinal tissue.
- Simple direct measurement of collagen content is insufficient to quantitate tissue fibrosis.
- However, measurement of collagen morphometrics correlates with biochemical metrics of tissue fibrosis, including col1A1, col III gene expression, αSMA protein expression and histological scoring.
- SHG tortuosity and reticulation are independent of inflammatory gene expression.
- Collagen structural changes (as evidenced by increased tortuosity and reticulation) precede induction of αSMA protein and MF activation.

- Future work will evaluate these image analysis tools for assessment of strictures in human Crohn's disease.