Introduction: Timely reconstruction of peripheral nerve injuries has better prognosis. But there is no non-destructive means to assess the degree of damage in crush or stretch injuries. Hence, the clinician must simply wait and observe for recovery, thus losing the best opportunity for optimum repair.1,2 What is needed is a reliable, accurate, non-destructive assay of the degree of internal nerve damage that can be performed early in the post-injury period and that is predictive of the injured nerve’s capacity and likelihood to spontaneously recover.

Second Harmonic Generation (SHG) via multiphoton microscopy is a novel modality that can produce high-resolution imaging of tissues comprising of non-centrosymmetric molecules like collagen.3 With peripheral nerve being rich in collagen-I, we validated the structural inferences of collagen damage as seen in the SHG images with conventional histology and immunohistochemistry (for myelin).4 To validate the prognostic utility of SHG, we have now performed a longitudinal study to examine recovery and correlate such recovery with SHG images of the nerve damage. SHG can non-destructively image the interior of an intact peripheral nerve and yield information about the degree of fascicular disruption after stretch injury that is predictive of the prognosis for spontaneous recovery.

Methods: With the approval of IACUC, low and high stretch injuries were induced on the right median nerve of 10-month old female Sprague-Dawley rats. A customized caliper-based microclamp was developed in-house for this process. The left nerve was maintained as a sham control to set off the effect of this apparatus. A functional assay, the grip strength test, was performed prior to injury and periodically afterwards until sacrifice (1, 3, 8, and 12 wks post-injury). Rats were sacrificed upon surgery (day 0), 1, 3, 8, and 12 wks after surgery (n=10/timepoint). 5 of each were subjected to low strain (LS) injury and the other 5 to high strain (HS) with potential to cause lasting functional damage and supraphysiologic damage.

A naïve control group was sacrificed at baseline (n=5) apart from age-matched controls sacrificed 12 wks from baseline (n=17). The functional measure was first obtained from the age-matched group. Then low stretch injury was inflicted on the right median nerves of a homogenous selection of 8 of these rats, high stretch injury was induced on 7, while the left median nerves were maintained as sham controls. 2 were maintained as naïve controls.

Upon sacrifice, median nerves were harvested, fixed in formalin, and embedded in paraffin. 8μm thick sections were used to stain for collagen structure (hematoxylin and eosin (H&E)) and imaged under the brightfield microscope. Contiguous sections were deparaffinized studied under the multiphoton microscope via SHG imaging. Findings from conventional histology, SHG imaging, and functional indicators were mapped out quantitatively, with strong potential for quantitative scoring in the near future.

Results: There is a clear demarcation in nerve response to LS vs. HS. Collagen fibers are linear and well-organized in naïve controls as compared to the sham. Sham nerves show mild fascicular disorganization at the point of clamp placement. This helps distinguish the effect of clamping against the injury. Upon LS, the endoneurium is composed of disorganized collagen fibers that are wavy and non-linear. HS injury affects both the endoneurium and epineurium. There are significant tears and ruptures apart from collagen fiber disorganization, undulations, and increase in fiber thickness. Shredding of the collagen fibers is also common. 1wk after injury, regenerative mechanisms set in. 3wks after injury, Schwann cells have mildly increased in the LS group alongside the appearance of digestion chambers. Collagen fibers are still mostly disorganized but signs of restructuring are evident. With the nerves exposed to HS, there is a large increase in the Schwann cell population that is much greater than the cell response to a LS injury. There are several digestion chambers and the collagen structure remains just as disorganized and injured as day 0, likely indicating that a neuroma-in-continuity is forming, rather than restitution of functional nerve tissue. At 8wks, collagen structure in the LS group begins to normalize, although the undulating pattern of the collagen fibers persists, to a degree. Also, Schwann cell content remains just as high as the previous timepoints with an increase in the number of digestion chambers. The HS group at 8 weeks retains the abundance of Schwann cells, minimal digestion chambers, enlarged nodes of ranvier, and sustained structural disruption, indicating the arrest of regenerative processes. By 12wks, the LS nerves show nearly complete structural recovery while the HS group shows early regeneration efforts, but failure to restore the normal, longitudinal, organized structure of the nerve fascicles that would be consistent with return of function. This structural pattern in peripheral nerve recovery from the SHG imaging was compared with contiguous hematoxylin and eosin-stained images using light microscopy in order to correlate and validate the SHG image interpretation (Fig. 1)

The functional assay reflects these trends, albeit imperfectly. (Fig. 2) With a steep drop in functionality a week after injury owing
to damage to the nerve and pain from surgery, there is some recovery by 3wks. This recovery plateaus by 8wks with a mild drop in functionality, and picks up again by 12wks. These trends are consistent between the LS and HS groups but the recovery in activity is slightly improved in the LS group. When compared with baseline, the LS group has a slightly higher activity value while HS has suffered a lasting loss in functionality, albeit mild. At 12wks, both injury groups are distinctly lower in absolute activity levels than the age-matched naïve group.

**Discussion**: The SHG images of peripheral nerve structure completely correlate with conventional histology. The inferences drawn on structural recovery post low and high stretch injury run consistently between the two. The functional grasp test, while indicating trends on nerve recovery, does not consistently correlate. This functional test, like all others, is confounded, to a degree, by subject volition and compensation by non-affected (e.g. ulnar-innervated) muscle groups. There is the anticipated latency between reparative cellular activity and its functional manifestation as improved grip5, though our work reinforces unreliability and foibles of functional tests which could cause incorrect treatment decisions to be made.

**Significance**: Clinicians currently use functional tests to assess nerve damage and the need for timely surgical intervention due to the lack of a non-destructive tool that truly reflects nerve structure. It explores the possibility of SHG as a clinically translatable, minimally invasive, and non-destructive tool to provide an accurate picture of nerve structure.

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Figure 2. Rate of change in activity from before surgery