# 11 Second-Harmonic Generation Imaging of Self-Assembled Collagen Gels

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# **11.1 Introduction**

The biological molecules that exhibit second-harmonic generation (SHG) include fibrillar collagens, myosin, microtubules, silk, and cellulose [1-4]. Laser scanning microscopy (LSM) allows for noninvasive and nondestructive three-dimensional imaging of the SHG signal from biological samples possessing second-harmonic-generating molecules [5-9]. As an optical signal, SHG is uniquely sensitive to the spatial organization of generating dipoles [10-14], allowing for quantitative and selective structural characterization of second-harmonic-generating tissue. Numerous optical and structural parameters have been derived from the SHG signal, from which inferences can be made about tissue structural, compositional, optical, and mechanical properties [15-26].

This chapter focusses on SHG signal imaging studies of hydrogels composed of acid-solubilized type I collagen. Self-assembled silk and cellulose scaffolds that generate second-harmonic signal have also been characterized [4,17,18,27]. The prevalence of studies utilizing collagen hydrogels originates from the extremely important role and ubiquitous presence of collagen in connective tissues throughout the body. Collagen is the most common protein in the body, comprising 6% of body weight and ~25–33% of the total protein mass [28]. Self-assembled collagen hydrogels are not only useful *in vitro* models to study cell-matrix interactions [29–32], cellular modulation of wound healing [8,33–41], fibrosis [42–47], and microstructure–mechanics relations [21,22,48–52], but they also serve as starting scaffolds for many tissue-engineering experiments and applications [42,43,45,53–55].

Cell-seeded collagen hydrogels are particularly amenable to analysis by three-dimensional LSM, including SHG imaging, since the tissues may be imaged nondestructively at any time during *in vitro* culture [5,24,56–61]. Collagen self-assembly is controlled by polymerization conditions that influence polymer aggregation, creating gels of varied microstructures and network properties [21,22,62–68]. The cells seeded in or on such hydrogel scaffolds create tissue constructs that change dynamically due to force interactions between cells and the surrounding scaffold, and by proteolysis and new matrix deposition. Dynamic remodeling of cell-seeded collagen gels may be tracked in four dimensions by assembling *z*-stacks of SHG image frames from tissues at different locations and culture time points. Other optical signals, such as reflectance [69], optical coherence [70], one- and two-photon fluorescence (TPF) [56,71], and coherent anti-Stokes Raman scattering (CARS) [72] may be imaged simultaneously or nearly on a multimodal platform, increasing the structural, biochemical, and optical information derived from the imaged tissue regions and allowing the study of interactions between the signal-producing species within the tissue. SHG imaging of acellular and cell-seeded self-assembled gels is a powerful technique to address the fundamental questions regarding tissue mechanics and cell behavior within a three-dimensional matrix environment.

# 11.2 Background

## **11.2.1** Collagen Structural Properties

Over 20 unique types of collagen have been described, including the fibril-forming collagens (types I, II, III, V, and XI) [63,73]. Each of these fibril-forming collagens posses a similar noncentrosymmetric structure that is required for both SHG and for self-assembly into supramolecular aggregates and, finally, an entangled network. The three properties of SHG, self-assembly, and resistance to tension arise from the unique primary structure of fibril-forming collagens. This structure consists of repeats of the triple amino acid sequence glycine-X-Y, where X and Y are most frequently proline and hydroxyproline, respectively [73]. These repeats comprise ~10% of the collagen monomer sequence and enable the formation of left-handed helices, termed alpha chains tightly coiled with about three amino acids per turn. The three alpha chains interact with each other to form a right-handed, coiled-coil triple helix. This triple helical procollagen molecule is capped by nonhelical propeptide regions that promote solubility and whose presence and enzymatic removal are both necessary for fibrillogenesis to occur [65]. Procollagen molecules are synthesized near the endoplasmic reticulum and must be packaged and secreted by the Golgi apparatus before extracellular initiation of fibrillogenesis. Procollagen N- and C-peptidase are extracellular enzymes that cleave the 15 and 10 nm-long N and C propeptides, respectively, yielding a collagen monomer ~300 nm long and 1.5 nm wide [73]. Following procollagen cleavage, the collagen monomer contains only the helical region capped by nonhelical telopeptide regions that consist of 10-25 amino acids, and are important in cross-linking and in directing monomer packing and fibrillogenesis. The interchain spacing within the triple helix is ~0.286 nm, close enough for hydrogen bonding, hydrophobic interactions, and interchain cross-links to stabilize the monomer. After the conversion of procollagen into collagen monomers, fibrillogenesis may occur. The process is an entropy-driven self-assembly that may occur in a cell-free environment modulated by physical variables (pH, temperature, ions) or in living tissue where cell-secreted molecules and enzymes may modulate fibrillogenesis [73].

## 11.2.2 Collagen Gel Self-Assembly

Collagen for the *in vitro* construction of collagen gels is derived by soaking collagen-rich tissues (such as rat-tail tendons or calf skin) in acetic acid for several days, followed by dialysis to concentrate the solubilized, triple-helical collagen monomers [74]. The resulting soluble collagen contains few intramolecular cross-links, though aggregates of 5–17 monomers may exist in soluble form [63,73]. Raising the temperature, pH, and ionic strength to physiological levels typically initiates self-assembly, in which collagen fibrils form via lateral and linear fusion of monomer aggregates. Fibril length, diameter, and aggregation into fibers are affected by polymerization, pH, temperature, and ionic strength [21,22,62–68,75]. Selfassembly results in the formation of a collagen gel, that is, an entangled network of highly hydrated collagen fibrils surrounding fluid-filled pores. The gelation process proceeds with an initial lag phase, during which the monomer aggregates initiate fusion, followed by a rapid growth phase, and eventual plateau. The mechanical and optical properties of the gel change with gelation time in a similar, sigmoidal shape. For example, gel turbidity (a measure of light scattering) increases as

$$x = 1 - \exp(-Z_n t^n), \tag{11.1}$$

where x is the mass fraction of precipitated collagen (linearly related to turbidity), Z is a rate constant, *t* is the gelation time, and *n* is a constant related to collagen fiber nucleation [65]. Bulk shear modulus of the gel also increases rapidly after a lag and prior to a plateau value [73]. The collagen self-assembly process is hypothesized to occur through simultaneous nucleation and linear growth of fibrils [73]. In this model of collagen fibrillogenesis, collagen monomers exist in equilibrium with small, soluble aggregates of 5-17 monomers, termed microfibrils. The existence of these purported microfibrils is supported by x-ray diffraction and electron microscopy measurements of collagen fibrils, which suggest that fibrils exist with quantized diameters, of integer multiples of ~4 nm. Self-assembly proceeds by both lateral and axial accretion of these microfibrillar subunits [76]. Increasing ion concentration or decreasing temperature or pH tends to favor the lateral aggregation of microfibrils, leading to increased fibril diameters [73,77]. The physical parameters that increase fibril diameter tend to delay fibrillogenesis and prolong the lag phase, during which nucleation is the predominant process [63]. Other extracellular matrix constituents present during collagen self-assembly may also modulate the collagen fibril and network structure. Proteoglycan and glycosaminoglycan binding to collagen may either delay or accelerate fibrillogenesis, thus affecting the collagen fibril diameter. Hyaluronic acid and decorin tend to decrease the diameter of fibrils formed in their presence [23], whereas dermatan sulfate binding favors the formation of thicker fibers [78]. The physical and chemical parameters can alter in vitro fibrillogenesis that affects collagen fiber dimensions and, given a limited concentration of collagen monomers, can vary the fiber number density and therefore the pore size of the formed collagen network, thus impacting network mechanics. The final result of collagen self-assembly and fibrillogenesis is an ordered and hierarchical array of collagen monomers, forming an entangled biopolymer network of fibrils and fibril bundles.

The fibril level of organization consists of arrays of monomers that are ordered in an axially staggered pattern in which molecules are stacked and staggered by one-quarter of the molecular length, or about 68 nm. This staggered array allows for interchain cross-link formation, and results in native fibrils 20–500 nm wide and up to 1 cm long [79] displaying *D*-banding: 68 nm wide bands that stripe the collagen fibril, apparent in metal-stained fibrils and resulting from the quarter-staggered array of monomers [80]. Bundles of fibrils form collagen fibers, typically 1–20  $\mu$ m wide; bundles of fibers form thicker fascicles that form bundles within tendons [81]. Between the monomer and the assembled collagen unit, physical and biological factors alter the assembly process. Importantly, collagen structure and cross-link content affect the mechanical properties on the level of single-collagen fibers, collagen networks, and bulk tissue. Knowledge of the mechanisms of collagen fibrillogenesis allows control over the formation of collagen-containing tissues *in vitro* and *in vivo*.

#### **11.2.3 Optical Properties of Collagen Gels**

SHG signal intensity depends upon the square of the incident laser intensity, the square of local collagen concentration, and, due to SHG coherence, on the spatial organization and scattering properties of the generating collagen dipole arrays [10–12,14,16]. SHG intensity,  $I_{SHG}$ , may be expressed as

$$I_{\rm SHG} = 16\pi (\omega^2 / n_{\omega}^2 n_{2\omega}^2 c^2) \kappa S_{2\omega}^2 |d_{\rm eff}^2| I_{\omega}^2, \qquad (11.2)$$

where  $\omega$  is the fundamental (laser frequency),  $n_{\omega}$  is the refractive index at the fundamental frequency,  $n_{2\omega}$  is the refractive index at the second-harmonic frequency, c is the speed of light,  $\kappa$  is a function of particle size,  $S_{2\omega}$  is the second-harmonic backscattering coefficient (SHG scattering cross-section),  $d_{eff}$  is the effective second-order nonlinear susceptibility, and  $I_{\omega}$  is the laser intensity within the focal region [14,16,82,83].

The SHG signal from collagen gels arises from a coherent/quasi-coherent component due to direct detection of the generated signal and an incoherent component due to the detection of multiply-scattered signal. SHG signal depends, therefore, on collagen gel-scattering properties that influence the incoherent component as well as fibril size, aggregation, and orientation that influence the coherent signal component [84]. Collagen possesses a high refractive index ( $n \sim 1.5$ ) [14], and thus scatters a significant amount of light in an aqueous environment (n = 1.33). Single-photon scattering by collagen occurs according to Mie theory, in which collagen fibrils smaller than the wavelength of incident light tend to scatter equally in the forward and backward direction. Collagen fibers and close-packed fibrils that approach and exceed the wavelength of scattered light possess increasing scattering cross-sections, and are predominantly forward scattering [14]. Similarly, SHG from a point source smaller than  $\lambda_{20}/10$ (or about 40 nm) radiates more homogenously, whereas SHG from a cluster of harmonophores larger than  $\lambda_{2\omega} \sim 400$  nm, the second-harmonic wavelength, produces almost entirely forward-generated second harmonic [10,14,84]. Theoretical and experimental work suggest that collagen fibril aggregates can generate significant backward-generated second-harmonic signal due to relaxed phase-matching conditions imparted by interfibrillar spacing roughly equal to the coherence length in the backward direction [85]. Hence, SHG is primarily forward generated by large fibers and fibril bundles  $>\lambda_{200}$ , but significant backward-generated SHG may result from small fibrils  $\ll \lambda_{2\omega}$  with spacing on the order of the backward coherence length, which for collagen SHG is less than 7 µm [85]. At a typical LSM optical resolution of ~450 nm, collagen fibers and fibril aggregates at least two pixels wide will be 900 nm >  $\lambda_{2\omega}$ , resulting in primarily forward SHG. Smaller fibrils, however, may produce up to 25% backward-generated SHG, augmenting signal detection in the epidirection [84,85].

Turbidity of a collagen gel solution has been shown to vary linearly with collagen concentration for low concentrations of collagen (<4 mg/mL) [56]. Not surprisingly, due to the reliance of SHG signal on backscattering events, a linear dependence of SHG on acellular collagen gel concentration has also been reported [14]. Thus, it is clear that the bulk optical properties of nonlinear scattering signals from a collagen-rich tissue depend on collagen concentration, microstructure, and fiber size [86].

#### **11.2.4 Image Processing**

Generally speaking, quantitative information from SHG images can result from analysis of signal levels or from analysis of image textural and spatial features. To the extent that second-harmonic image parameters are sensitive to collagen fiber and network structure (e.g., fiber, orientation, or network anisotropy), the parameters may be used as indices that track microstructure–mechanics relationships. The interpretation of some quantitative image parameters is unambiguous. For example, the diameter of collagen fibers or network pores measured manually or algorithmically from SHG images is a direct assessment of fiber and network structure. The mean SHG signal, on the other hand, is a function of fiber shape, orientation, image area fraction, bulk gel collagen content, and bulk gel scattering

properties. Furthermore, some parameters such as mean SHG signal depend upon objective numerical aperture, detector sensitivity, laser parameters, and other external factors [1–3]. For such instrument-dependent parameters, absolute values are relatively meaningless without proper instrument calibration, and information is best gleaned by analysis of trends or by taking a ratio to effectively normalize the parameter, with the ratio being independent of instrument parameters. For example, careful calibration and optical setup allows for the measurement of forward-to-backward signal ratios [23,25,27].

# 11.3 SHG Imaging of Acellular Collagen Gels

# 11.3.1 Introduction

Acellular type I collagen gels are ideal constructs to study the relationship of collagen fiber and network structural characteristics to SHG signal. These gels are pure and polymerization conditions may be varied independently of collagen concentration to control the aspects of fiber and network microstructure. At low concentrations (<~9 mg/mL), the fiber network is sparse enough that individual fiber features can be resolved as well as network features. SHG image parameters correlate with fiber and network structural features, with implications for using SHG images to estimate bulk mechanical properties.

## 11.3.2 Quantification of Collagen Fiber Shape from Second-Harmonic Images

Altering the polymerization, pH, and temperature of collagen hydrogels significantly influences the gel microstructure and mechanical properties at a given collagen concentration. In the experiments described below, gel collagen concentration was kept constant at 4 mg/mL and polymerization was varied between 4°C and 37°C, or between pH 5.5 and 8.5. It was found that increasing pH or temperature tends to result in longer, thinner collagen fibers, a reduced pore area fraction and size, and an increased pore density. These characteristics are visible in both SHG and TPF images (Figures 11.1a through 11.1d, 11.1i through 11.1l) and scanning electron microscopy (SEM) images (Figures 11.1e through 11.1h, 11.1m through 11.1p) of acellular collagen gels. SHG signal to noise is larger than that of TPF signal, a difference that is likely reflective of the quadratic SHG versus linear TPF concentration dependence, and also on the generally weak autofluorescence signal from poorly cross-linked collagen. Therefore, incoherent and homogeneous emission allows the fiber cross-sections to be clearly seen by TPF signal, whereas the coherent nature of SHG disallows signal generation from dipoles within collagen oriented parallel to the laser propagation. SEM images have a higher intrinsic resolution and reveal that collagen fibers are actually closely packed bundles of fibrils, with especially large bundles containing many fibrils at the lower temperature and pH polymerization conditions. Fiber diameter varies across the polymerization conditions because of differences in the number of fibrils per fiber rather than large changes in fibril diameter.

The measurements of fiber diameter from SHG and SEM images reveal a linear correlation (Figure 11.2), although the diameters measured from SEM images tend to be smaller (due to dehydration of the fibrils during sample preparation). Small diameter fibers are visible in SHG (Figures 11.1a, 11.1b, 11.1i, and 11.1j) and SEM images (Figures 11.1e, 11.1f, 11.1m, and 11.1n) of gels polymerized at the lower temperature and pH values, sometimes independent of larger diameter fibers and sometimes emanating from the splayed ends of large diameter fibers. However, SHG and SEM images show that large diameter fibers dominate the space-filling characteristics of these gels. With increasing polymerization temperature and pH, the hydrogels display a finer and more homogeneous network of fibers.

## 11.3.3 Effects of Collagen Fiber Size on Second-Harmonic Signal

In acellular collagen hydrogels, in which collagen is the only significant scattering component, backward-detected SHG signal primarily results from scattering of forward-generated second-harmonic photons and from backward-generated SHG from small fibrils (diameters ~10% of  $\lambda_{2\omega}$ ). The



**FIGURE 11.1** (See color insert.) Simultaneously collected SHG (blue signal) and TPF (green signal) images of 4 mg/mL acellular collagen hydrogels polymerized at various temperatures (a-d) and pH values (i-l), with corresponding SEM images (e-h, m-p). The scale bars are indicated in the figure. (Reprinted from *Biophys J.*, 92, Raub, C. B. et al., Noninvasive assessment of collagen gel microstructure and mechanics using multiphoton microscopy, 2212–2222, Copyright 2007; *Biophys J.*, 94, Raub, C. B. et al., Image correlation spectroscopy of multiphoton images correlates with collagen mechanical properties, 2361–2373, Copyright 2008, with permission from Elsevier.)



**FIGURE 11.2** Correlation of manual fiber diameter measurement averages from SHG images ( $d_{SHG}$ ) and SEM images ( $d_{SEM}$ ) of acellular collagen gels polymerized at pH 5.5, 6.5, 7.5, and 8.5, or temperatures 4°C, 14°C, 24°C, and 37°C. The linear best-fit lines are shown, with slope and  $R^2$  indicated in the figure. The error bars are standard deviation. (Reprinted from *Biophys J.*, 92, Raub, C. B. et al., Noninvasive assessment of collagen gel microstructure and mechanics using multiphoton microscopy, 2212–2222, Copyright 2007; *Biophys J.*, 94, Raub, C. B. et al., Image correlation spectroscopy of multiphoton images correlates with collagen mechanical properties, 2361–2373, Copyright 2008, with permission from Elsevier.)



**FIGURE 11.3** (a) Mean segmented SHG signal versus polymerization temperature of acellular collagen gels. (b) Mean segmented SHG signal versus polymerization pH of acellular collagen gels. (c) Mean segmented SHG signal plotted versus average fiber diameter measured from SHG images,  $d_{SHG}$ , for acellular gels polymerized at pH 5.5, 6.5, 7.5, and 8.5, or temperatures 4°C, 14°C, 24°C, and 37°C. The error bars are standard error of the mean. (Reprinted from *Biophys J.*, 92, Raub, C. B. et al., Noninvasive assessment of collagen gel microstructure and mechanics using multiphoton microscopy, 2212–2222, Copyright 2007; *Biophys J.*, 94, Raub, C. B. et al., Image correlation spectroscopy of multiphoton images correlates with collagen mechanical properties, 2361–2373, Copyright 2008, with permission from Elsevier.)

dependency of epi-detected SHG signal on collagen fiber size can be seen in Figures 8.3a and 8.3b, Q1 for 4 mg/mL acellular collagen gels polymerized from 4°C to 37°C and pH 5.5–11, respectively. The SHG signal has been segmented to exclude void regions, so that the measurements represent average signal values only from collagen. Plotting the segmented SHG signal versus fiber diameter shows a direct, linear correlation for both varying pH ( $R^2 = 0.96$ ) and temperature ( $R^2 = 0.85$ ) polymerization conditions, with an offset attributable to the differing detector gain (Figure 11.3c). The effect of increasing mean fiber diameter is to increase the scattering cross-section of the fiber as well as bulk scattering within the tissue, which is thus able to scatter more forward-directed SHG photons into the epi-configured detectors.

## 11.3.4 Effects of Acellular Collagen Gel Concentration on Second-Harmonic Signal

While the concentration dependence of second-harmonic signal scales with the square of generating dipole concentration, changes in the bulk collagen concentration of acellular gels may increase both the average density of collagen within signal-containing pixels and the relative volume fraction of collagen within the gel. The effect of increasing fiber number density can be seen in SHG images for fine- (Figures 11.4a through 11.4c) and coarse-structured gels (Figures 8.4d through 8.4f) with low Q1 collagen concentrations (1.5-9 mg/mL). For these collagen gels, SHG signal mean intensity (Figure 11.4g) and area fraction (Figure 8.4h) increase linearly with collagen content. The linearity of the signal OI increase is robust to changes in collagen fiber morphology observable from the SHG images and may be attributed simply to changes in collagen fiber number density and concomitant decreasing of void volume fraction, rather than increased fibril packing within pixels, which would introduce a nonlinear (second-order) dependence of the signal on collagen concentration. The scattering coefficient within collagen gels is expected to increase by ~2.9 cm<sup>-1</sup> per 1 mg/mL of collagen, from 4.3 cm<sup>-1</sup> at 1.5 mg/ mL, and 26 cm<sup>-1</sup> at 9 mg/mL [87], and may contribute to linear increases in SHG signal intensity with collagen concentration. A similar linear trend in SHG area fraction versus collagen concentration (Figure 11.4h) suggests that for these acellular, low-density collagen gels, the microstructure determines SHG image parameters.



Q2 FIGURE 11.4 Simultaneously collected SHG (blue signal) and TPF (green signal) images of acellular collagen hydrogels polymerized at pH 8.5 (a-c) or 14°C (d-f) and collagen concentrations of (a,d) 1.5 mg/mL, (b,e) 4.5 mg/mL, and (c,f) 9 mg/mL. (g) Normalized SHG signal versus normalized collagen concentration for the fine- and coarse-structured gels, with linear best-fit lines shown. (h) SHG signal image area fraction versus collagen concentration for fine- and coarse-structured gels, with linear best-fit lines shown. The error bars represent standard deviation. The data markers are indicated in the figure legend. The scale bar represents 50 μm.

## 11.3.5 Effect of Collagen Fiber Orientation on SHG

Several studies have shown through theoretical and experimental methods that SHG depends upon the orientation of collagen monomers aggregated into fibrils and fibers with respect to both the laser polarization angle and the laser propagation direction [3,10–14,25,84,88]. Specifically, for a fibril perpendicular to the laser propagation direction, SHG is maximized when the dipoles within the fibril are aligned parallel to the incident electric field and is minimized when the dipoles are perpendicular. The coalignment of dipoles and the electric field allows for a maximum nonlinear polarization. This orientation dependence of SHG has been used to characterize the in-plane orientation of collagen fibrils since it has been shown that the dipoles within collagen align with the fibril long axis [13].

The orientation dependence of SHG signal from collagen fibrils, though a useful structural parameter, can interfere with accurate structural characterization of a collagen network from a single SHG image or image stack since fibrils would possess variable SHG intensity depending upon fibril orientation. For circularly polarized laser illumination, however, SHG signal does not depend upon fibril–dipole orientation within the image plane [14]. However, there still exists an axial (out-of-plane) dependence, in which SHG is maximized from fibrils perpendicular to the laser propagation direction (i.e., perpendicular to the image plane), and is minimized from fibrils parallel to the laser propagation direction (i.e., perpendicular to the image plane). The following discussion addresses the axial dependence of SHG signal in collagen

gels, showing that SHG from in-plane fiber cross-sections is more intense that from fibers orientated with long axes perpendicular to the image plane.

The theoretical orientation dependence of SHG signal was determined by utilizing the previously described expression for SHG intensity,  $I_{2\omega}$  [86,89]

$$I_{2\omega} = \frac{p}{n_{2\omega}n_{\omega}^{2}}(I_{\omega})^{2} d_{eff}^{2} \left(\int_{z_{0}}^{z_{0}+L} \frac{e^{i\Delta kz}}{1+iz/z_{R}}dz\right)^{2},$$
(11.3)

where in this case, p is a lumped term of fundamental constants and beam parameters,  $n_{2\omega}$  and  $n_{\omega}$  are the index of refraction at the SHG and fundamental wavelengths,  $I_{\omega}$  is the laser intensity at the focal point,  $d_{eff}$  is collagen's orientation-dependent effective second-order nonlinear susceptibility,  $z_R$  is the Rayleigh distance, and  $\Delta k$  is the phase mismatch. Assuming that p,  $n_{\omega}$ ,  $n_{2\omega}$ ,  $z_R$ , and  $\Delta k$  remain constant over any fiber orientation, the ratio of SHG intensities from fibers nearly perpendicular ( $I_{2\omega}^{\perp}$ ) versus fibers nearly parallel ( $I_{2\omega}^{\perp}$ ) to the laser propagation direction is then

$$I_{2\omega}^{\perp}/I_{2\omega}^{=} = (d_{eff}^{\perp})^{2}/(d_{eff}^{=})^{2}, \qquad (11.4)$$

where  $d_{eff}^{\perp}$  and  $d_{eff}^{=}$  are the effective second-order nonlinear susceptibilities of collagen fibers perpendicular and parallel to the laser propagation direction, respectively. For parallel polarized laser light, the nonlinear susceptibility  $d_{eff}$  is [90,91]

$$d_{eff} = 3d_{16}(\cos\beta\cos\delta - \cos^3\beta\cos^3\delta) + d_{22}\cos^3\beta\cos^3\delta, \qquad (11.5)$$

where  $\beta$  is the angle of the (assumed randomly oriented) fiber axis with respect to the fundamental electric field and  $\delta$  is the angle between the fiber axis and the imaging plane. The case of circularly polarized laser light corresponds to allowing  $\beta$  to vary between 0° and 360°, and averaging  $d_{eff}^2$  over all values of  $\beta$  for a specific value of  $\delta$  between 0° (fiber perpendicular to laser propagation) and 90° (fiber parallel to laser propagation). The ratio  $d_{22}/d_{16}$ , necessary to calculate  $d_{eff}$  was estimated to be ~2, based upon previous studies in collagen [89]. In this study, experimentally determined ratios of  $I_{2\omega}^{\perp}/I_{2\omega}^{=}$  were compared to these calculations to determine their reasonableness. This ratio was calculated by carrying out a fiber segmentation, using TPF signal, into circular (out-of-plane) fiber cross-sections *c*, and elliptical (in-plane) fiber cross-sections *e*, and then determining the ratio of colocalized SHG signal in these segmented fiber regions.

Fibers parallel and perpendicular to the image plane are visible in TPF images (see Figures 11.1a, and 11.1i) and can be segmented after thresholding based upon particle circularity (Figure 11.5a, inplane fibers based upon TPF signal from coarse-structured gel similar to that of Figure 11.1a; 8.5b, Q1 out-of-plane fibers based upon TPF signal). In contrast, only fibers with scattering interfaces more or less parallel to the image plane (and perpendicular to the laser propagation direction) produce strong backward-detected SHG signal (see Figures 11.1a, 11.1i).  $\text{TPF}_c/\text{TPF}_c \pm \text{SE}$  was  $1.1 \pm 0.1$  for the pH 5.5 condition and  $0.90 \pm 0.05$  for the pH 6.5 condition;  $\text{SHG}_c/\text{SHG}_c \pm \text{SE}$  was  $3.1 \pm 1.3$  for the pH 5.5 condition and  $2.9 \pm 0.8$  for the pH 6.5 condition (Figure 11.5c).

The squared effective nonlinear susceptibility of collagen was calculated using Equations 11.3 through 11.5 and was plotted as a function of  $\delta$ , the angle of the fiber axis with respect to the image plane (Figure 8.5d). A ratio of 3 for  $SHG_e/SHG_e$ , for example, corresponds to  $d_{eff}^2$  values of 2.64 and 0.88 for fibers ori- Q1 ented at  $\delta = 10^{\circ}$  and 63°, respectively (Figure 11.5d). These example values were chosen to show that the theoretical estimation of the ratio of  $d_{eff}^2$  based upon fibers tilted at shallow versus steep angles tends to predict an SHG intensity ratio similar to experimentally determined values.



**FIGURE 11.5** (a) Mask of in-plane-oriented collagen fiber cross-sections, taken from a TPF image of an acellular collagen hydrogel. (b) Mask of out-of-plane-oriented collagen fiber cross-sections, taken from a TPF image of an acellular collagen hydrogel. (c) The ratio of noise-subtracted SHG and TPF signal from the signal-containing regions masked in (a) (elliptical particles, *e*) and (b) (circular particles, *c*). (d)  $d_{eff}^2$ , in arbitrary units was estimated for various angles of tilt of the collagen fiber axis with respect to the laser propagation direction. Fibers at 10° and 63° of tilt are diagrammed with respect to the laser focal region (gray). (Reprinted from *Biophys J.*, 94, Raub, C. B. et al., Image correlation spectroscopy of multiphoton images correlates with collagen mechanical properties, 2361–2373, Copyright 2008, with permission from Elsevier.)

The 1:1 ratio of TPF signal from fibers predominantly parallel versus transverse to the image plane confirms the isotropic angular distribution of TPF generation in collagen gels. In contrast, one would expect greater backward-detected SHG from fibers parallel to the image plane, both because of scattering of forward-generated SHG at the fiber bottom interface [14], and because of the collagen dipoles' optimal orientations with the laser propagation direction [73]. Indeed, a 3:1 ratio of SHG intensity from fibers roughly parallel versus roughly transverse to the image plane was measured, which is consistent with theoretical considerations using a lower numerical aperture (NA) [92]. A higher NA [1.3] objective was used to collect the SHG and TPF images, introducing a component of the electric field transverse to the image plane, which would change the SHG angular power distribution from fibers aligned with the z-axis. Specifically, these fibers should emit forward-generated SHG at a steeper angle ( $\sim 40-47^{\circ}$ ) from the z-axis compared to the lower NA case, but no backward-generated SHG due to the Guoy phase shift and destructive interference{}. Fibers aligned parallel to the laser propagation should exhibit lower  $d_{eff}$  $(d_{eff}^{=} < d_{eff}^{\perp})$  as well as reduced single and multiple scattering of SHG photons from the highly forwarddirected SHG emission compared to fibers oriented perpendicular to the laser propagation direction. Although a higher NA objective provides additional electric field contributions to SHG signal, the forward-directed generation and lack of scattering interfaces in transverse collagen fiber sections could explain the observed threefold difference in epi-detected SHG signal.

From Equation 11.5, a ratio of SHG signal intensities was calculated by estimating values of  $d_{eff}$ , which depend on the orientation of collagen dipoles with respect to the incident electric field. This calculation

is valid if the phase mismatch  $\Delta k$  and the index of refraction remain constant regardless of fiber orientation. In fact, collagen's birefringence of  $\Delta n \sim 0.003$  (where *n* along the collagen monomer's long axis > *n* along the short axis) is assumed to be negligible. This seems a valid assumption since the calculation is compared to an experimental SHG signal averaged over an ensemble of collagen fibers only roughly aligned. Furthermore,  $\Delta k$  may be assumed constant since the SHG interaction volume (roughly the focal volume dimensions, ~200 nm lateral × 600 nm axial) is smaller than the mean fiber diameter and length in these cases, and can be assumed to be completely filled when centered on a collagen fiber regardless of fiber orientation. For an incident wavelength of 780 nm,  $\Delta k \sim 0.48 \,\mu$ m, with a corresponding coherence length of 6.5  $\mu$ m, allowing efficient SHG from ~3  $\mu$ m thick bundles of collagen fibrils.

In summary, second-harmonic-generating dipoles within a collagen fibril or fiber tend to align with the fibril long axis. For linearly polarized laser light, SHG depends on the angle between the laser's electric field polarization direction and the fibril long axis. For circularly polarized incident light, this dependence is removed. However, SHG still depends upon the angle of the fibril long axis with respect to the laser propagation direction, with maximal signal perpendicular and minimal signal parallel to the propagation direction. In essence, a circularly polarized LSM with SHG capability will capture the signal from collagen oriented within the plane of the image, but with less and less signal from collagen oriented increasingly perpendicular to the image plane. While an image of SHG signal from a collagen gel may thus underestimate the true collagen content, most collagen structures seem to emit detectable SHG signal. A superimposition of SHG and TPF signals from the same region of an acellular collagen gel typically reveals the full cross-sectional structure of the collagen network.

### 11.3.6 Quantification of Collagen Network Architecture from Second-Harmonic Images

The pores within collagen gels allow diffusion, facilitate cell migration, and affect gel mechanical properties. Collagen network pores larger than the optical resolution set for a given imaging experiment may be quantified from SHG images. The collagen network could become so dense that 100% of image pixels contain collagen SHG signal, in which case, an alternative method to determine the pore characteristics would have to be used, but for most acellular collagen gels, the collagen network is sparse enough that pores may be characterized from SHG images. The pore characteristics were quantified from SHG and TPF signal of collagen gels polymerized at 4 mg/mL and pH 5.5-8.5 (Figures 11.6a and 11.6b). The average pore size and pore area fraction tended to decrease, whereas pore number density increased with increasing polymerization, pH, as the available collagen tended to form more numerous, thinner fibers at higher pH values. Particle analysis of thresholded SHG images produced a mean pore size  $\pm$ SE of 81.7  $\pm$  3.7 mm<sup>2</sup> for the pH 5.5 condition, decreasing ~90% to 7.8  $\pm$  0.4 mm<sup>2</sup> for the pH 8.5 condition (Figure 11.6a). As expected, the number density of pores increased ~3.2-fold from 7.1  $\pm$  0.2 to 23.0  $\pm$  0.6 per 1000 mm<sup>2</sup> (Figure 8.6b). The **Q1** trends in pore characteristics were confirmed by particle analysis of thresholded SEM images that demonstrated a similarly trending decrease in pore size and increase in pore number density with increasing polymerization pH. From this analysis, it appears that SHG images of acellular gels yield direct information about pore characteristics that correspond to measurements from SEM images. In case TPF images contain additional pore information due to the orientation independence of TPF signal, pores were quantified from thresholded SHG signal alone and also from thresholded SHG and TPF signals combined. The particle analysis method of measuring pore characteristics is fairly robust, as analysis of the combined SHG and TPF signal-masked images showed little change in the pore measurements.

## 11.3.7 Determining Mechanical Relationships in Acellular Gels from Second-Harmonic Images

In two separate studies described below, an attempt was made to correlate SHG and other image parameters to bulk gel mechanical properties from shear and indentation tests. In the first study, varying



**FIGURE 11.6** (a) Mean pore size, determined from particle analysis from SHG and combined SHG + TPF images, of acellular collagen gels polymerized at pH 5.5, 6.5, 7.5, and 8.5. (b) Mean pore number density, determined from particle analysis from SHG and combined SHG + TPF images, of acellular collagen gels polymerized at pH 5.5, 6.5, 7.5, and 8.5. The error bars represent standard deviation. (Reprinted from *Biophys J.*, 94, Raub, C. B. et al., Image correlation spectroscopy of multiphoton images correlates with collagen mechanical properties, 2361–2373, Copyright 2008, with permission from Elsevier.)

the polymerization temperature of 4 mg/mL collagen hydrogels systematically changed the fiber volume fraction and space-filling characteristics as well as bulk shear moduli, measured by rheology. The shear moduli *G*′ and *G*″ were found to correlate positively with SHG image fraction for these gels (Figure 11.7a), although the correlation between the shear moduli and SHG image fraction is most linear for the 14–37°C polymerization conditions. In this case, the collagen fibers polymerized at 4°C formed an extremely weak, sparse gel, with most fibers not entangled and therefore not contributing to an elastic stress response. In contrast, the shear moduli correlate negatively with mean-segmented SHG signal for 4 mg/mL collagen gels with polymerization temperature-controlled microstructure (Figure 11.7b). Since all gels contained collagen at 4 mg/mL, gels with larger diameter fibers contained fewer (though larger with brighter SHG signal) fibers and larger pores, linking the shear moduli, SHG signal, and SHG image area fraction. This study was an initial attempt to correlate the mechanical properties with SHG image parameters, in an attempt to understand the origins of the shear moduli values. It was concluded that the network space-filling characteristics, specifically of the pores between collagen fibers, play an important role in determining mechanical properties, and that SHG image area fraction is one way to describe collagen network space-filling characteristics.

The second study was designed to attempt to explain bulk gel mechanics with a microstructural model and SHG image-derived microstructural parameters. Acid-soluble collagen hydrogels consist of an uncross-linked, tightly entangled network of semiflexible (but rather rod-like) polymer chains (i.e., fibers), and as such, their viscoelastic properties may be modeled, employing only a handful of microstructural input parameters [93]. Specifically, the storage modulus G' scales as  $G' \sim \rho^{7/5} L_p^{-1/5}$ ; (8.6) where  $\rho$  is the density of polymer contour length per unit volume and  $L_p$  is the chain's persistence



**FIGURE 11.7** (a) Average shear moduli (storage modulus, G'; loss modulus, G'') versus average SHG image area fraction from 4 mg/mL acellular collagen gels polymerized at temperatures 4°C, 14°C, 24°C, and 37°C. (b) Average shear moduli (storage modulus, G'; loss modulus, G'') versus mean segmented SHG image intensity from 4 mg/mL acellular collagen gels polymerized at temperatures 4°C, 14°C, 24°C, and 37°C. (Reprinted from *Biophys J.*, 92, Raub, C. B. et al., Noninvasive assessment of collagen gel microstructure and mechanics using multiphoton microscopy, 2212–2222, Copyright 2007, with permission from Elsevier.)

length. A network mesh size,  $L_m$ , relates to  $\rho$  as  $\rho = L_m^{-2}$ . A fiber's persistence length is proportional to its bending modulus, which for collagen scales with the fourth power of fiber diameter, d [94,95]. Recasting the scaling relationship of Equation 8.6 in terms of mesh size and fiber diameter,  $G' \sim L_m^{-14/5} d^{-4/5}$  (11.7). To determine if SHG images can provide structural data that scales appropriately with G', the mesh size and fiber diameter were estimated from SHG images (Figure 11.8a) in two ways: using particle analysis and manual fiber diameter measurements ( $P_{PA}$  and  $d_{SHG}$  for fibers and pores, respectively), and using image correlation spectroscopy, which create characteristic pore and fiber diameters,  $P_{ICS}$  and  $d_{ICS}$ , based upon signal periodicity in the SHG image [22]. The data from SHG images were input into the scaling relationship and a plot of log G' versus log ( $L_m^{-14/5} d^{-4/5}$ ) was generated. The correlation using the particle analysis and hand-measured parameters is good ( $R^2 = 0.93$ ). The best-fit slope is of the order one ( $m \sim 0.84$ , Figure 11.8b). One conclusion to draw from this correlation is that mesh size and fiber diameter, parameterized from SHG images, may explain most of the variation in storage modulus of these acellular collagen gels, and that the gels behave as entangled networks of semiflexible fibers.

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**FIGURE 11.8** (See color insert.) (a) Combined SHG + TPF (SHG, blue signal; TPF, green signal) image of acellular collagen hydrogel showing examples of measurements of collagen fiber diameter and network mesh size. (b) Log–log plot of the scaling relationship of the storage modulus versus mesh size,  $L_m$ , and fiber diameter, *d*, estimated from SHG images of collagen gels polymerized at temperatures 4°C, 14°C, 24°C, and 37°C. The estimates were from image correlation spectroscopy ( $P_{1CS}$  for mesh size;  $W_{1CS}$  for fiber diameter), or particle analysis and manual image measurements ( $P_{PA}$  for mesh size;  $d_{SHG}$  for manual image measurements). The linear best-fit slopes and  $R^2$  values are indicated in the figure. (Reprinted from *Biophys J.*, 94, Raub, C. B. et al., Image correlation spectroscopy of multiphoton images correlates with collagen mechanical properties, 2361–2373, Copyright 2008, with permission from Elsevier.)

#### 11.3.8 Summary: SHG Imaging of Acellular Collagen Gels

In the studies described above, acellular collagen gels were polymerized at a range of concentrations (1.5-9 mg/mL), temperatures (4-37°C), and pH values (5.5-8.5). The segmented signal intensity, image area fraction, average fiber diameter, and average mesh size were measured from SHG images of the gels. In some cases, a scaling relationship from semiflexible network theory was applied, showing good correspondence to experimental data inputs (average fiber diameter, mesh size from SHG images, G' from rheology). While this correspondence shows the sensitivity of SHG imaging to mechanical properties, there are limits to the microstructural information captured by SHG signal. Most notably, the smallest structures that can be measured from SHG images are determined by the optical resolution of the system. Structural information on the order of single pixels can still be obtained through careful measurement of optical parameters—such as signal orientation dependence with respect to polarizers, or forward-to-backward signal ratios. Second, SHG signal decays exponentially with penetration depth into the tissue [1,2,96]; so, the interrogated microstructure must be within a resolvable distance (typically ~150 µm to several mm). Third, SHG is specific only for certain structural proteins, most notably collagen and myosin [3,97-100]; so, SHG imaging will fail to capture mechanically relevant matrix components that do not emit, such as elastin, proteoglycans, cells, and noncollagenous tissue structures. The matrix components that do not emit second harmonic may be characterized through other imaging modalities and signals, such as TPF [21,22,26,59,101,102]. Acellular collagen gels, however, possess a uniform microstructure and are free of noncollagenous matrix components, which allow for very thorough structural characterization of these gels from SHG images alone. Tissue-engineering experiments utilize collagen gels as matrix scaffolds for cells, which typically remodel the gel through stress generation, protease activity, and new matrix deposition. SHG imaging, especially in conjunction with other nonlinear optical signals, can provide detailed information about the dynamic changes during culture of cellularized collagen gels.

# 11.4 SHG Imaging of Cellularized Collagen Gels

## 11.4.1 Introduction

The studies described below use MPM imaging and mechanical testing to study floating cellularized Q4 collagen gels, which is contracting to less than 5% of their original volume, attain physiological collagen, and cell densities. During the contraction process, cellularized gels exhibit microstructure-dependent trends in mechanical and optical properties, and these dynamic changes may be captured through MPM imaging and analysis of the SHG signal. The analysis of microstructure-mechanics relationships in these simple engineered tissues is important to understand how engineered tissues develop and how cell-induced matrix remodeling occurs in vitro. Cellularized collagen gels were prepared in a method similar to that used to make acellular gels at pH 6.5 (coarse-structured gels) and 8.5 (fine-structured gels), except that 50,000 normal human lung fibroblasts (NHLFs), passages 3-7 were added per milliliter of a 4 mg/mL collagen solution. The gels were polymerized in 24-well plates at room temperature (24°C) for 1 h and were then pH equilibrated with excess culture media. After overnight tissue culture to allow fibroblasts to adhere and spread within the collagen gels, the constructs were released from the wells and were placed in floating culture in Petri dishes half-filled with culture media. These floating gels were cultured in standard conditions for up to 15 days, during which time the gels were periodically removed for imaging and mechanical testing. To monitor the cell location and interactions with collagen, the imaging wavelength was set to 780 nm, and SHG signal from collagen was collected at 390 nm, while TPF signal from endogenous fluorophores [56,96] within the fibroblasts was collected at 500-550 nm.

## 11.4.2 Effect of Gel Contraction on SHG Images

Fine and coarse gels retained distinct microstructures, revealed in MPM images by SHG signal, even after significant cell-induced contraction (Figures 11.9a through 11.9c, fine-structured gels; 11.9d through 11.9f, coarse-structured gels). Matrix defects and holes appear in the SHG images from denser cellularized gels, typically adjacent to areas with cellular TPF signal (Figure 11.9f). The final collagen concentration approached approximately 200 mg/mL for the most contracted gels, with a final volume of ~11  $\mu$ L (Figure 11.9g). Collagen mass content and total cell content increased during the culture period, in a trend clearly visible from the coregistered SHG and TPF images.

Interestingly the mean SHG signal intensity from both fine- and coarse-structured gels increased linearly with collagen concentration (Figure 11.9h, signal and concentration normalized), identically to acellular gels (Figure 11.4g). The linear trend of SHG signal with bulk collagen concentration may be interpreted as a function of increased collagen volume fraction and multiple backscattering of SHG signal within the gel.

## 11.4.3 SHG Image Texture Simulation

To determine whether SHG image parameters could accurately assess the collagen network microstructure from cellularized gels varying in concentration over two orders of magnitude (~4–200 mg/mL), simulated textural images with well-defined numbers of overlapping "collagen fibers" were constructed to recreate structural features from the SHG images of cellularized gels. Using the simulations of image texture, the relationship between image parameters and collagen fiber number density could be determined and trends could be compared between the simulation and SHG images.

The textural features of images of the SHG signal from coarse-structured collagen gels were simulated using a MATLAB<sup>®</sup> routine. The constructed images were meant to simulate the images of a randomly oriented collagen fiber network, to determine the precise relationship of robust, gain-independent image parameters of fiber number density. Collagen fiber segments within the MPM image plane were simulated as two-dimensional elliptical Gaussian functions. The length and width of the fiber segments



**FIGURE 11.9** MPM images of (a–c) fine-structured and (d–f) coarse-structured cellularized collagen gels at three stages of contraction during floating culture. The estimated collagen concentration for each gel is indicated, and days of culture. SHG signal (blue) and TPF signal (green) reveal collagen and fibroblasts. The bar represents 50  $\mu$ m. (g) Measured gel volume and the corresponding estimated collagen concentration estimate versus culture time. (h) Normalized SHG signal versus normalized collagen concentration for fine- and coarse-structured cellularized gels in various stages of contraction during free-floating culture. The linear best-fit slopes and  $R^2$  values are indicated. (Reprinted from *Acta Biomater*, 6, Raub, C. B. et al., Predicting bulk mechanical properties of cellularized collagen gels using multiphoton microscopy, 4657–4665. Copyright 2010, with permission from Elsevier.)

were distributed normally, with mean and standard deviation determined from n = 50 line-segment measurements from SHG images of real collagen gels. Gaussian peak intensity was directly related to the length and width, so that larger fiber segments proportionally possessed more intense signal. Furthermore, the fiber edges were defined where the signal fell to  $1/e^2$  times the maximum intensity of each Gaussian function. The simulated fiber areas and intensities were determined so that SHG images from cellularized gels at day 0 of culture would have similar mean intensity and signal area fraction to the simulated image of the corresponding fiber number density. Fiber orientations were distributed uniformly through 360° and were positioned at random locations within a 512 × 512 pixel matrix (the same size as the images from the cellularized gel imaging study). The intersecting fibers were allowed to superimpose, creating a linear relationship between the mean image intensity and fiber number density, as well as creating a reasonable approximation to the texture of SHG images from cellularized gels containing ~4–200 mg/mL collagen. To relate the simulated images to SHG images from cellularized gels, simulated images were assigned collagen concentrations equal to the number of Gaussian "fibers" in the simulation times with a scaling factor, with units of milligram per milliliter fiber number. The scaling factor was determined by counting the number of fiber segments in SHG images of cellularized gels at day 0 of culture. These SHG images were thresholded at the noise cutoff, despeckled as before to remove the remaining noise, and a binary opening algorithm was performed in ImageJ to isolate the adjacent fibers. Then, particle analysis was carried out in ImageJ to count particles larger than 1  $\mu$ m<sup>2</sup>. It was determined that the day 0 gel images contained 178 ± 34 fibers ( $\mu$  ± s.d.), and the averaged gel concentration was 6.4 mg/mL. Therefore, the scaling factor used for simulated images containing 200–5000 fiber segments was 0.0356 mg/mL/fiber. A second scaling factor ensured similar brightness of Gaussian ellipses in simulated images to collagen fibers in SHG images, both on an 8-bit [0–255] scale. The Gaussian ellipse brightness scaling was chosen such that the intensity of the sparsest simulated image equaled SHG image intensity from day 0 cellularized gels.

## 11.4.4 Multiphoton Image Parameters are Sensitive to Cellularized Gels Microstructure

To understand the microstructure–mechanics relationships of cellularized gels during cell-mediated matrix contraction, the image parameters were measured from SHG, TPF, and textural simulation images. Textural simulations of SHG images have a similar appearance to the SHG images of cellularized gels (Figures 11.10a through 11.10c, SHG images; 11.10d through 11.10f, texture simulation). Particle analysis of SHG images of cellularized gels on day 0 of culture revealed 178 ± 34 fiber segments (mean ± s.d.). On the basis of this measurement, simulation images containing 200–5000 fiber segments were assigned concentration values of 7–180 mg/mL. The visual comparison of SHG images and simulations shows a rough parity of texture and collagen fiber density for similar collagen concentrations.

Several signal and image parameters changed during the 16-day *in vitro* culture period. Trends from the textural simulations suggest that the functional form of SHG image parameters is largely due to



**FIGURE 11.10** (a-c) Images of SHG signal from cellularized gels, and (d-f) simulated images from a randomly oriented fiber network of similar texture to the SHG images. The SHG images are from cellularized gels at three stages of contraction during floating culture, and the simulated images are of roughly corresponding collagen fiber density. The estimated collagen concentration for each gel/image is indicated, and the days of culture. The bar represents 50 µm. (Reprinted from *Acta Biomater*, 6, Raub, C. B. et al., Predicting bulk mechanical properties of cellularized collagen gels using multiphoton microscopy, 4657–4665. Copyright 2010, with permission from Elsevier.)

Q1 changes in collagen fiber concentrations (Figure 8.11, SHG versus simulation). For example, SHG signal intensity is a linear function of collagen concentration (Figure 11.11a, solid line, m = 6.8 a.u./mg/mL,  $R^2 = 0.78$ ), which is corroborated by the linear relationship between image intensity and concentration in the simulated images (Figure 11.11a, dashed line, m = 6.9 a.u./mg/mL,  $R^2 = 1.0$ ).

SHG signal area fraction increases quickly to a plateau near 100% by ~60 mg/mL (Figure 11.11b, solid markers), and depends upon collagen concentration in a logarithmic fashion ( $R^2 = 0.66$  for the linear fit of ln(1-*area fraction*) versus *concentration*). This relationship is confirmed by the simulated signal area fraction (Figure 11.11b, open markers), which reaches a plateau near 100% by ~100 mg/mL ( $R^2 = 0.99$  for the linear fit of ln(1-*area fraction*) versus *concentration*). SHG signal intensity is not instrument independent and therefore, without calibration, has little microstructural information to provide, other than the expected linearity of the signal intensity with collagen concentration. SHG signal area fraction is more robust to instrument parameters, and suggests that in this experiment, cellularized gels containing ~60 mg/mL collagen contain very few "pores" or void regions with cross-sections larger than a single pixel (in this study, ~0.2 µm<sup>2</sup>). Therefore, an image analysis algorithm to extract pore information or image area fraction of the signal will be unhelpful to characterize the range of collagen microstructures over all gel contraction levels, and other robust image parameters should be sought.

The image parameters such as skewness and speckle contrast (SC) are gain independent and are thus more robust parameters to potentially characterize the structural features that impact bulk mechanics. In contrast to the linear intensity and log area fraction dependences, we find that the skewness of the image pixel histograms relates to collagen concentration in SHG images (Figure 11.11c) with a power-law dependence (SHG, exponent n = -0.6,  $R^2 = 0.90$ ; simulation, n = -0.5,  $R^2 = 0.99$ ). The SC of SHG images (Figure 11.11d, solid markers) and texture simulation images (Figure 11.11d,



**FIGURE 11.11** (a) Mean image intensity versus collagen concentration for SHG and texture simulation images. (b) SHG signal image area fraction versus collagen concentration for SHG and texture simulation images. (c) Mean image skewness versus collagen concentration for SHG and texture simulation images. (d) Mean speckle contrast versus collagen concentration for SHG and texture simulation images. (d) Mean speckle contrast versus collagen concentration for SHG and texture simulation images. Reference of the speckle contrast versus collagen concentration for SHG and texture simulation images. SHG values are filled circles; simulation values are open circles. The data points from SHG data represent an average of five images per gel.  $R^2$  coefficients for the linear best fits (a), logarithmic fits (b), and power-law fits (c,d) are given in the text. (Reprinted from *Acta Biomater*, 6, Raub, C. B. et al., Predicting bulk mechanical properties of cellularized collagen gels using multiphoton microscopy, 4657–4665. Copyright 2010, with permission from Elsevier.)

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open markers) scale similarly to skewness (exponent n = -0.6,  $R^2 = 0.90$  for SHG; n = -1.0,  $R^2 = 0.91$  for the simulation). Finding instrument-independent SHG image parameters that are sensitive to a range of cellularized gel concentrations is challenging, but the results above provide some evidence that SHG skewness and SC are sensitive to collagen network microstructure over that wide concentration range.

## 11.4.5 Skewness and SC of SHG and TPF Signals Predict *E* of Cellularized Gels

The multiphoton image parameters that serve as robust predictors of cellularized gel mechanical properties such as Young's modulus (*E*) must be gain independent and sensitive to the changes in collagen concentration, network microstructure, crosslinking, and changes in cellularity. The skewness and SC of SHG and TPF signals may possess the desired gain independence and structural sensitivity. To determine the strength of these image parameters in predicting *E*, multiple regressions were performed of log-transformed *E* values on log-transformed SHG, cell-derived TPF, and matrix-derived TPF skewness (*skew*) and SC parameters. The data points were from cellularized gels that were imaged with LSM for SHG and TPF signal, and were mechanically tested, after both *in vitro* culture and after glutaraldehyde cross-linking, which introduces fluorescent cross-links into the collagen network (Figure 11.12) [21]. The TPF signal was divided into cell and matrix components with a particle-based masking procedure. The cell-derived TPF parameters were found to covary with SHG parameters, being unable to



**FIGURE 11.12** (a) Nonlinear best-fit model for *E* on SHG signal skewness and matrix-derived TPF signal skewness. The markers indicate days in culture of the collagen gels and the presence of GTA cross-linking. The inset on the right is a log–log plot of the data. (b) Nonlinear best-fit model for *E* on SHG signal speckle contrast and matrix-derived TPF signal speckle contrast. The markers indicate days (notation "d1" for day 1) in culture of the collagen gels and the presence of GTA cross-linking. The inset on the right is a log–log plot of the data. Power-law best-fit exponents and *R*<sup>2</sup> values are indicated in the figure. *E* was averaged from five measurements per gel for *n* = 16 gels. (Reprinted from *Acta Biomater*, 6, Raub, C. B. et al., Predicting bulk mechanical properties of cellularized collagen gels using multiphoton microscopy, 4657–4665. Copyright 2010, with permission from Elsevier.)

explain the additional variation in *E*. We found that the relationship  $E \sim skew_{\text{SHG}}^{-1.0}skew_{\text{OF},\text{matrix}}^{-0.0}$  provided a best fit that explained the most variation in E (Figure 11.12a,  $R^2 = 0.80$ ). Similarly, the relationship  $E \sim SC_{SHG}^{-1.8}SC_{TF,matrix}^{-1.8}$  provided a best fit that explained the most variation in E (Figure 11.12b,  $R^2 = 0.83$ ). The observation of the linear and log-log plots of the multiple regressions shows that the nonlinear model using the skewness parameters tends to overestimate E of gels with sparse matrix (days 0-3, Figure 11.12a, inset), whereas the SC nonlinear model tends to overestimate E of uncross-linked gels Q1 cultured for 16 days (day 15, Figure 8.12b, inset).



## 11.4.6 Summary

Microstructural parameters change systematically during cell-mediated gel contraction: pores become smaller, fiber bundles become larger, and cells occupy holes in a dense three-dimensional collagen network. For cellularized collagen gels, SHG image parameters such as skewness and SC change with collagen fiber density in a predictable manner. Cellularized gel E can largely be predicted by the variation in SHG and matrix-derived TPF image parameters (skewness and SC), which depend upon collagen fiber and cross-link spatial patterns.

# **11.5 Conclusions**

MPM is a promising imaging tool that is currently being adapted for use with fiber-optic handheld probes and scanning modules. It has become the premier technique for imaging of living cells and cultures of excised and engineered tissues. There is a great deal of interest in the link between extracellular matrix microstructure and bulk tissue mechanical properties. Researchers are beginning to apply the knowledge of tissue microstructure derived from MPM for understanding the development of mechanical properties in tissues such as cellularized and acellular silk, the visceral pericardium [103], and the fibrous cap of atherosclerosis.

In this burgeoning research environment, there is a need to develop simple and robust methods for data mining of MPM images to extract all mechanically relevant information. Such mechanically relevant information includes the concentration of mechanically relevant species (e.g., collagen), the volume fraction of the species, the pore size of polymer networks, the fluorescent cross-link content of tissues, the diameter and length of fibers, and in general, the size and abundance of mechanically relevant structures, and the spatial distribution of species in three dimensions. This chapter focused on the measurement of mechanically relevant image parameters from SHG signals of acellular and cellularized gels.

Collagen gels of similar concentrations may nonetheless possess varied microstructure and resulting bulk mechanics. Signal area fractions, pore size measurements, and fiber diameter measurements from SHG images robustly and effectively estimate the aspects of collagen network microstructure that influence the bulk shear moduli.

MPM imaging of cellularized gels contracting through a range of collagen concentrations from 4 to 200 mg/mL revealed problems with the robustness of signal image fraction and pore size measurements. The optical resolution of LSM limits microstructural information extractable from SHG images, especially from concentrated gels (>60 mg/mL). The pores for these gels were smaller than the pixel resolution limit (~0.3 µm<sup>3</sup>) and could not be quantified. Nevertheless, SHG signal and image parameters are sensitive to a wide range of collagen network concentrations. MPM imaging of SHG signal provides an unparalleled noninvasive approach for studying microstructure-mechanics and cell-matrix interactions in collagen gel-based engineered tissues.

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