Supplementary information for "Nonlinear Optical Imaging to Evaluate the Impact of Obesity on Mammary Gland and Tumor Stroma", Le *et al.*

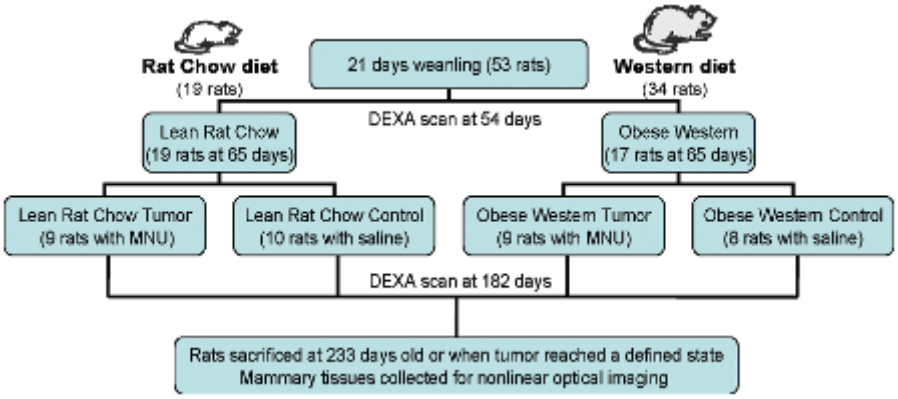


Figure S1. An early-onset diet-induced obesity breast cancer animal model. Of the 34 rats placed on the Western diet, 17 rats became obese. DEXA = dual-energy x-ray absorptiometry; MNU = methylnitrosourea.

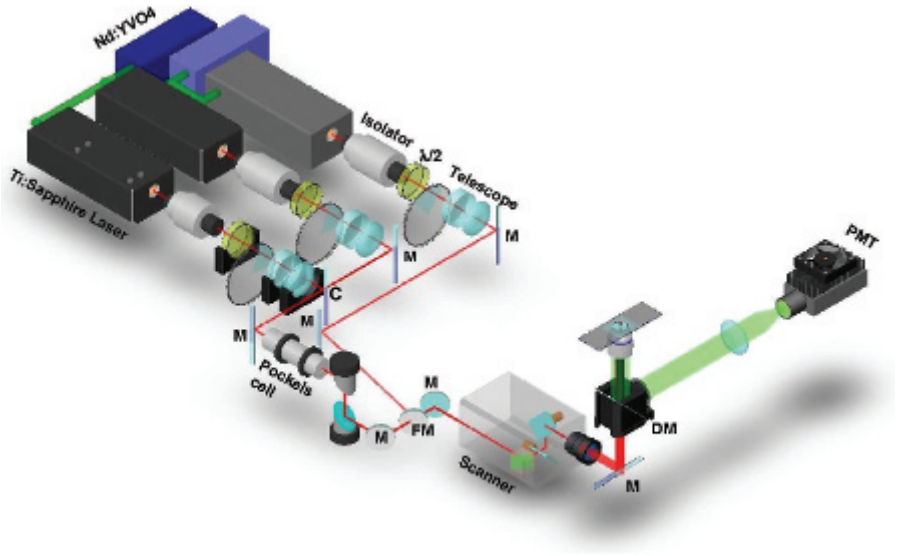


Figure S2. A multimodal multiphoton microscope that allows coherent anti-Stokes Raman scattering, second harmonic generation, and two-photon excitation fluorescence imaging on the same platform. C = combiner; DM = dichroic mirror; FM = flipper mirror; M = mirror; PMT = photomultiplier tube.

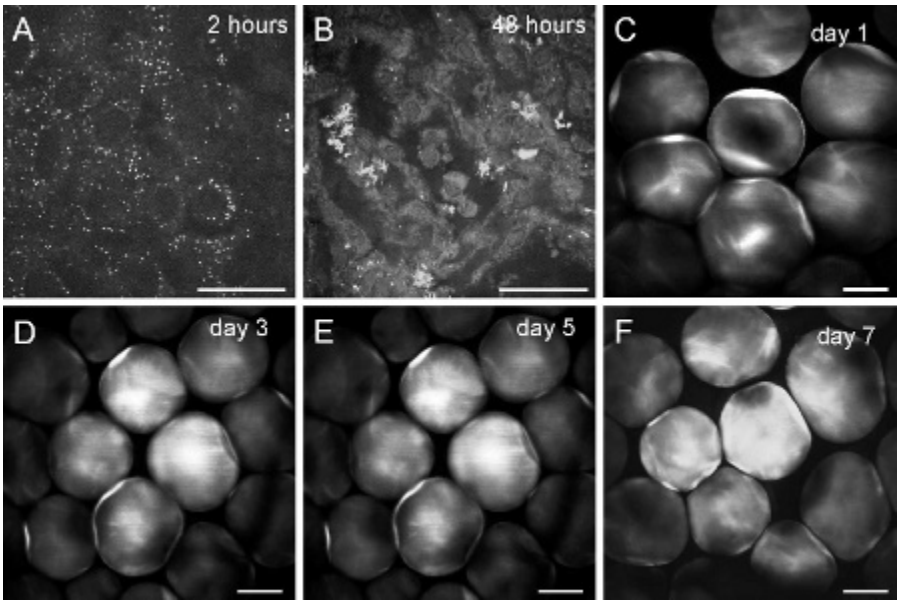


Figure S3. Stability of tumor cells of mammary tumor tissues and of adipocytes of mammary gland tissues in maintenance media. Tumor cells of mammary tumor tissues imaged with coherent anti-Stokes Raman scattering (CARS) at (*A*) 2 hours and (*B*) 48 hours after tissue collection. Adipocytes of mammary gland tissues imaged with CARS at (*C*) 1 day, (*D*) 3 days, (*E*) 5 days, and (*F*) 7 days after tissue collection. Scale bars = 25 μm.

Stability of Mammary Tumor and Mammary Gland Tissues

To analyze the stability of tumor cells of mammary tumor tissues and adipocytes of mammary gland tissues in maintenance media, we performed CARS imaging of tumor cells and adipocytes over time. During the first few hours after tissue collection, tumor cells were filled with lipid droplets, packed closely together, and had an average size of approximately 10 μm (Figure S3A). However, the lipid droplets in tumor cells disappeared rapidly over the next few hours. In addition, tumor cells shrunk to an average size of approximately 5 μm and gaps formed between cells at 48 hours after tissue collection (Figure S3B). In contrast, adipocytes remained stable for over 1 week in maintenance media (Figure S3, C–F). Learning from these observations, we imaged mammary tumor tissues immediately after collection or kept the tissues in liquid nitrogen for imaging at a later time. We found that tumor tissues stored in liquid nitrogen maintained the characteristics of freshly collected tissues (data not shown). We imaged mammary gland tissues immediately or up to 7 days after tissue collection. Mammary gland tissues were kept in maintenance media in an incubator set at 37°C and 5% CO_2. We found that freezing mammary gland tissues caused distortion to the shape of adipocytes (data not shown).

Labeling of Mammary Tumor Cells with Sp-DiOC18

Mammary tumor tissues were incubated in maintenance media mixed with Sp-DiOC18 (3 μg/mL final concentration; Molecular Probes, Eugene, OR) for 2 hours in a 37°C incubator with 5% CO_2. Tissues were washed three times and then incubated in maintenance media for 1 hour to remove excess DiOC18 before imaging with CARS and TPEF.

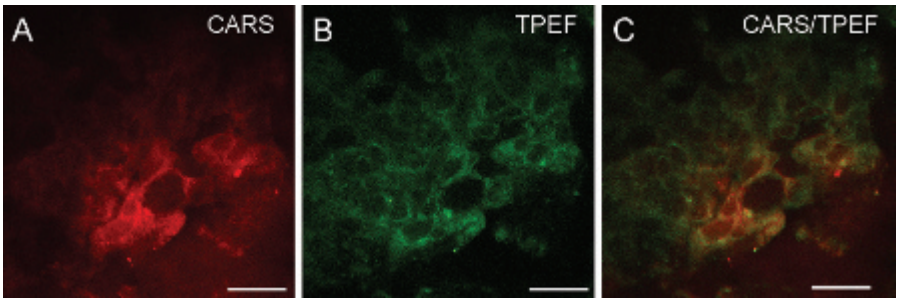


Figure S4. Mammary tumor cells labeled with DIOC18 and imaged with (*A*) coherent anti-Stokes Raman scattering (CARS) (*red*) and (*B*) two-photon excitation fluorescence (TPEF) (green). *C*, Overlay of CARS and TPEF images. Images taken with a 60× water immersion objective. Scale bars = 25 μm.

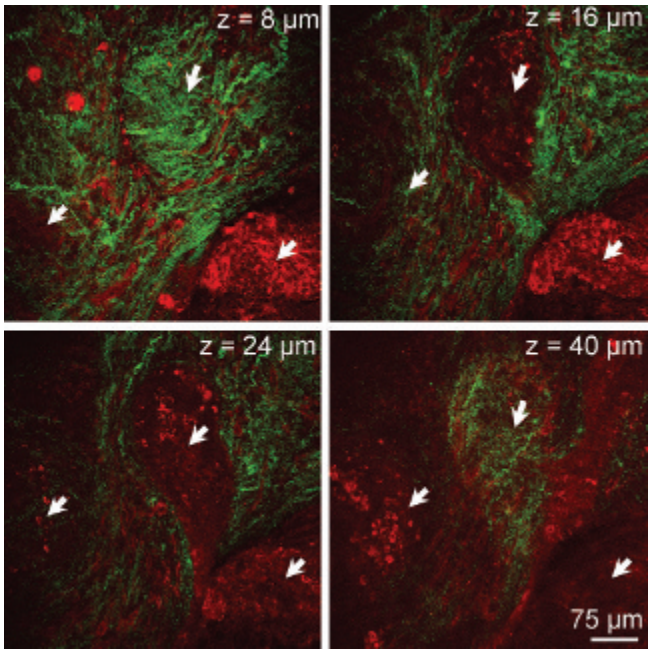


Figure S5. Imaging the orientation of collagen fibrils (*green*) with second harmonic generation and tumor mass with coherent anti-Stokes Raman scattering microscopy. *Arrows* point to tumor areas. Collagen fibrils appear to define the boundaries of the tumor mass. Images taken at the same area along the axial axis (z) using a 20× air objective.

Table S1. Evaluating the Impact of Obesity on Mammary Gland and Tumor Stromal Composition

Animal Groups (3 rats/group)	*Total Collagen Content (SHG intensity, × 10^9 arbitrary units)*	*Diameter of Adipocytes' LD (CARS imaging, µm)*
Lean rat chow		
LRC1	22.3	52.1 ± 10.9
LRC2	39.9	47.9 ± 7.8
LRC3	47.0	52.2 ± 9.4
Obese Western		
OW1	8.2	95.4 ± 13.7
OW2	7.2	85.1 ± 8.7
OW3	7.3	80.3 ± 8.9
Lean rat chow tumor		
LRCT1	0.6	NA
LRCT2	1.5	NA
LRCT3	2.7	NA
Obese Western tumor		
OWT1	19.5	NA
OWT2	16.2	NA
OWT3	29.9	NA

CARS = coherent anti-Stokes Raman scattering; LD = lipid droplet; NA = not available; SHG = second harmonic generation.

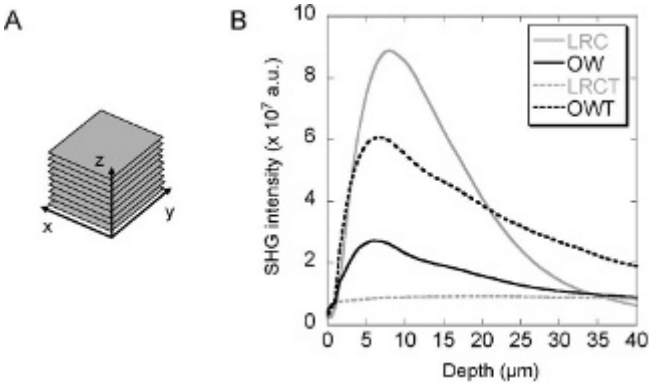
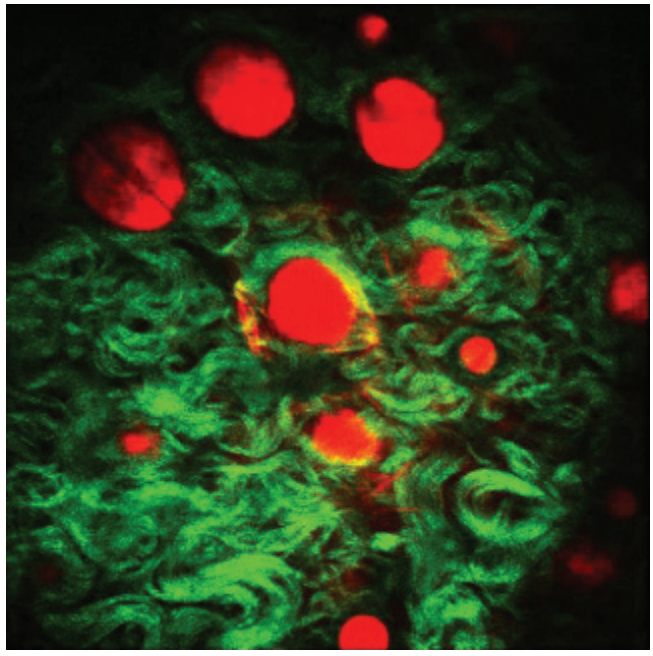
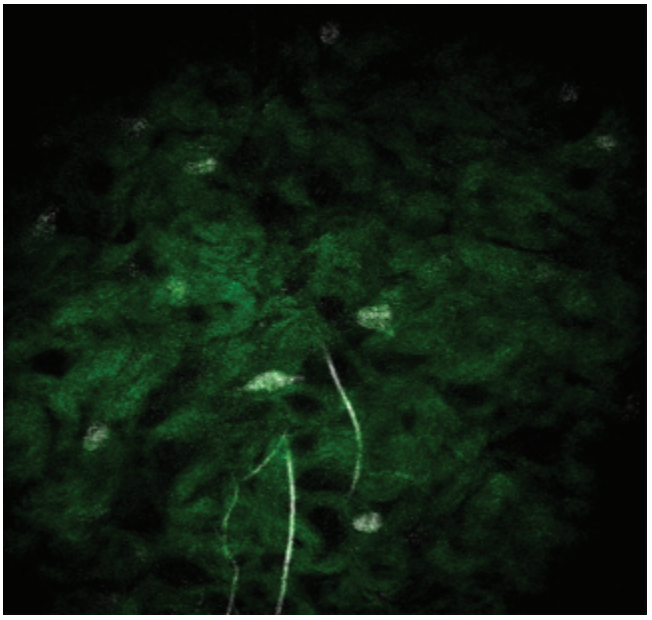


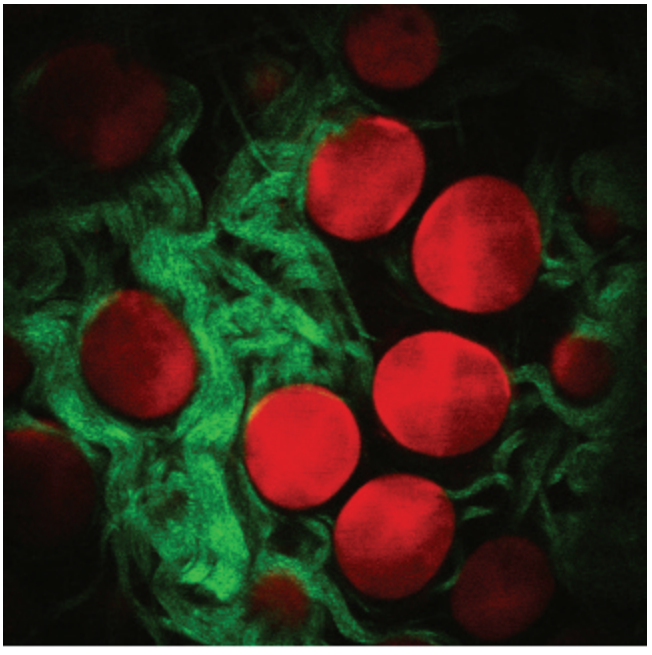
Figure S6. Analysis of mammary gland and tumor stromal composition of collagen fibrils type I. *A*, An analysis volume is defined with the dimensions of 250 µm (x) × 250 µm (y) × 40 µm (z), with z = 0 being the coverslip and tissue interface. For each analysis volume, 81 frames along the z-axis with a fixed step size of 0.5 µm were acquired. Nine different volumes were evaluated, and the total second harmonic generation (SHG) intensity from 729 frames was used to infer the mammary stromal collagen content of each animal. *B*, SHG intensity as a function of depth of one representative analysis volume in each animal. LRC = lean rat chow; LRCT = lean rat chow tumor; OW = obese Western; OWT = obese Western tumor.



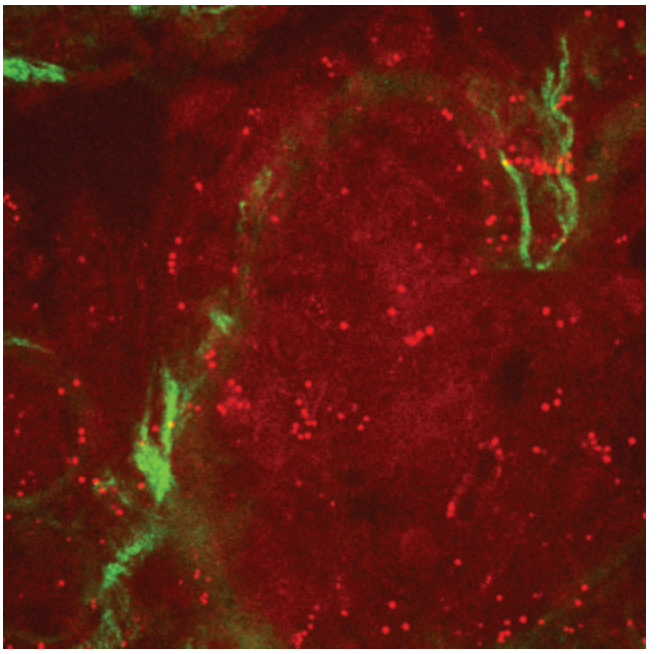
Supplementary Video S1. Three-dimensional imaging of adipocytes and blood capillaries with coherent anti-Stokes Raman scattering (*red*) and collagen fibrils type I with second harmonic generation (*green*) microscopy. The video is a collage of 30 frames taken at a 0.5 µm step size along the axial axis (z). A single frame at z = 6 µm is presented as Figure 1A.



Supplementary Video S2. Three-dimensional imaging of collagen fibrils with second harmonic generation (*green*) and fluorescein isothiocyanate conjugated isolectin B_4–labeled blood capillaries and activated macrophages with two-photon excitation fluorescence (*gray*) microscopy. The video is a collage of 150 frames taken at a 0.2 μm step size along the axial axis (z). A single frame at z = 6 μm is presented as Figure 1B.



Supplementary Video S3. Three-dimensional imaging of adipocytes with two-photon excitation fluorescence (*red*) and collagen fibrils type I with second harmonic generation (*green*) microscopy. The video is a collage of 30 frames taken at a 0.5 μm step size along the axial axis (z). A single frame at z = 8 μm is presented as Figure 1C.



Supplementary Video S4. Three-dimensional imaging of mammary tumor cells with two-photon excitation fluorescence (*red*) and collagen fibrils type I (*green*) with second harmonic generation microscopy. The video is a collage of 70 frames taken at a 0.5 μm step size along the axial axis (z). Three single frames are presented as Figure 1, D to F.