Supplemental Document

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Supplementary Information

Multidimensional quantitative characterization of the tumor microenvironment by multicontrast nonlinear microscopy

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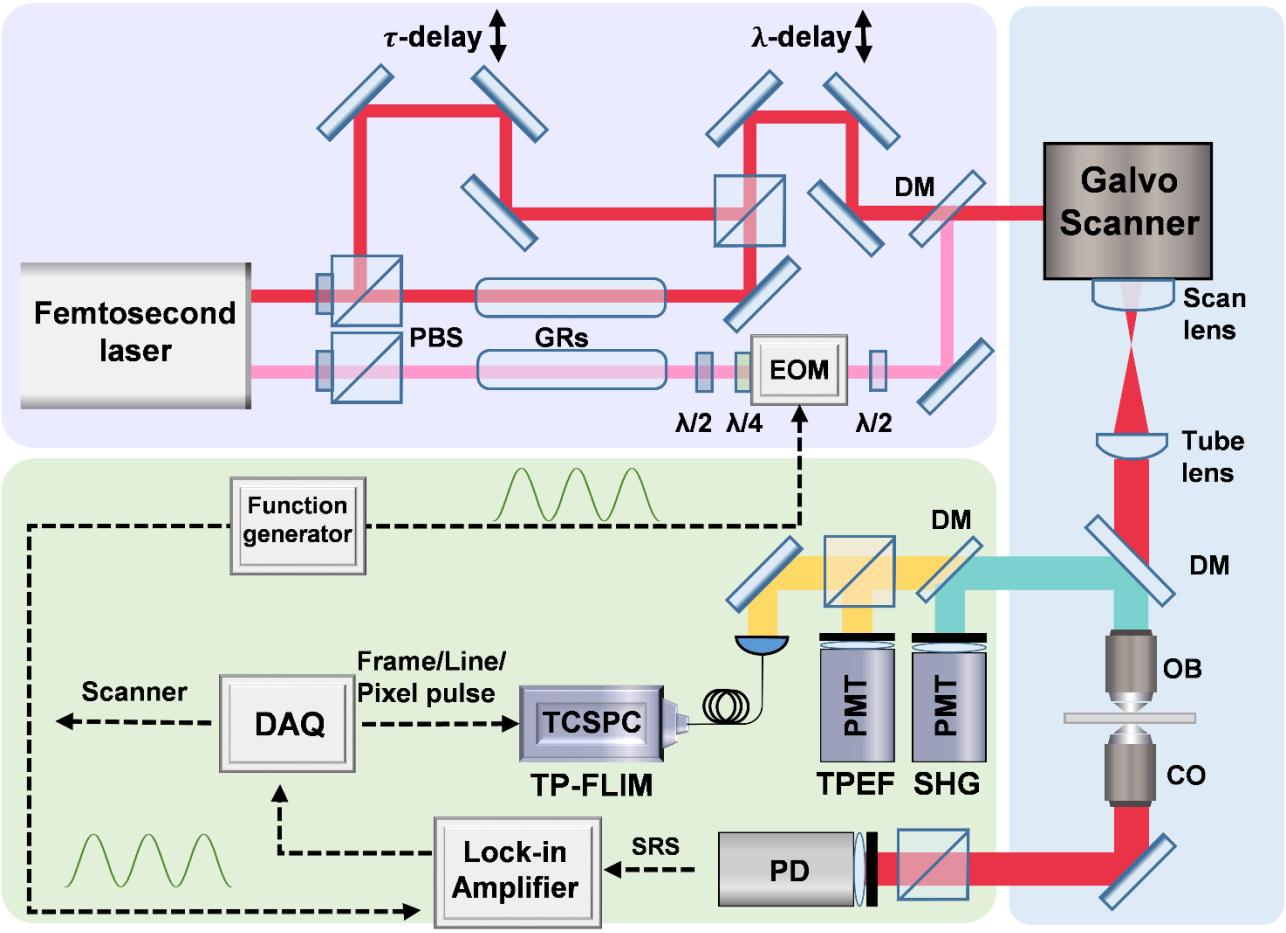
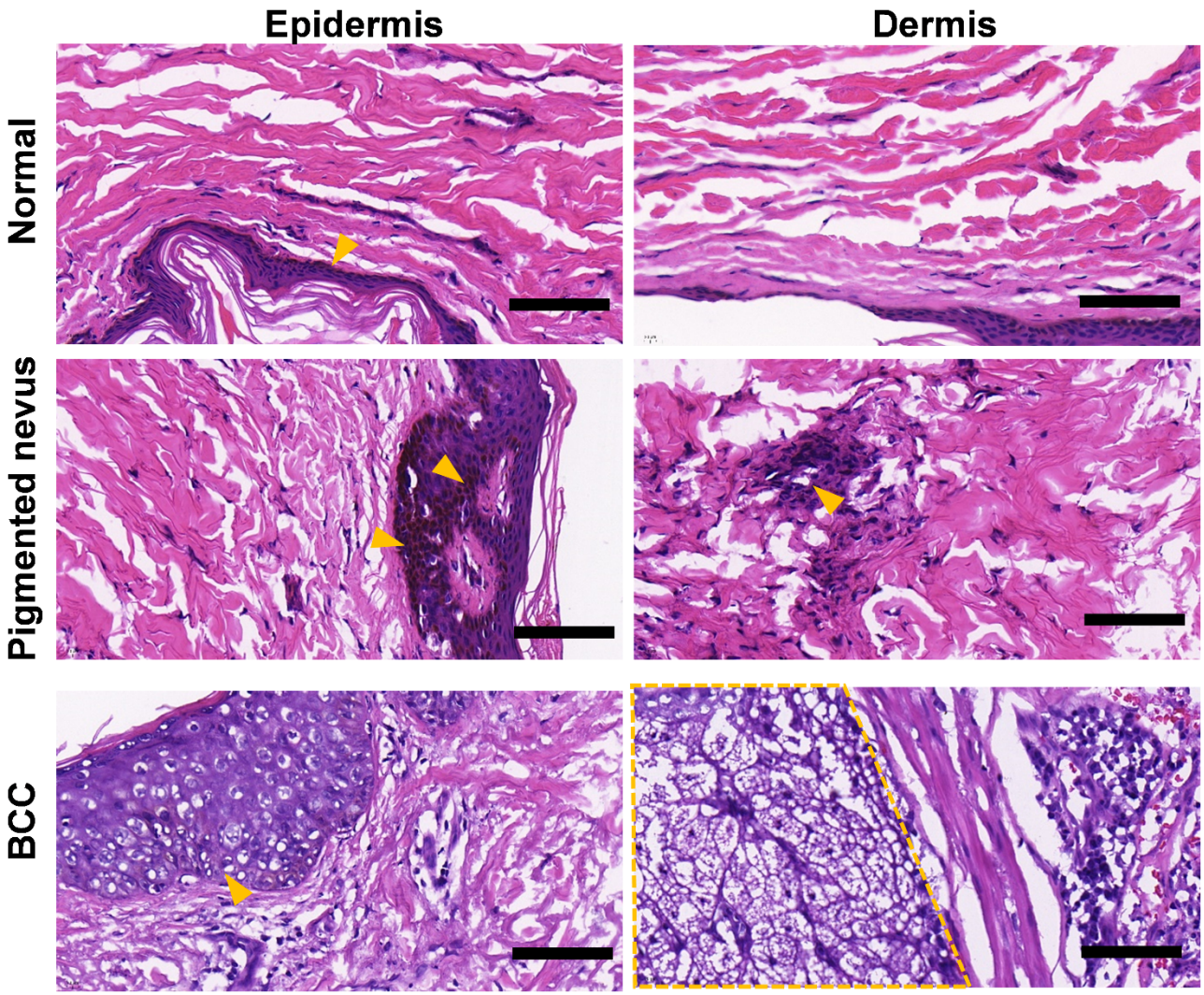
Supplementary Note 1 Simultaneous multidimensional nonlinear optical imaging system

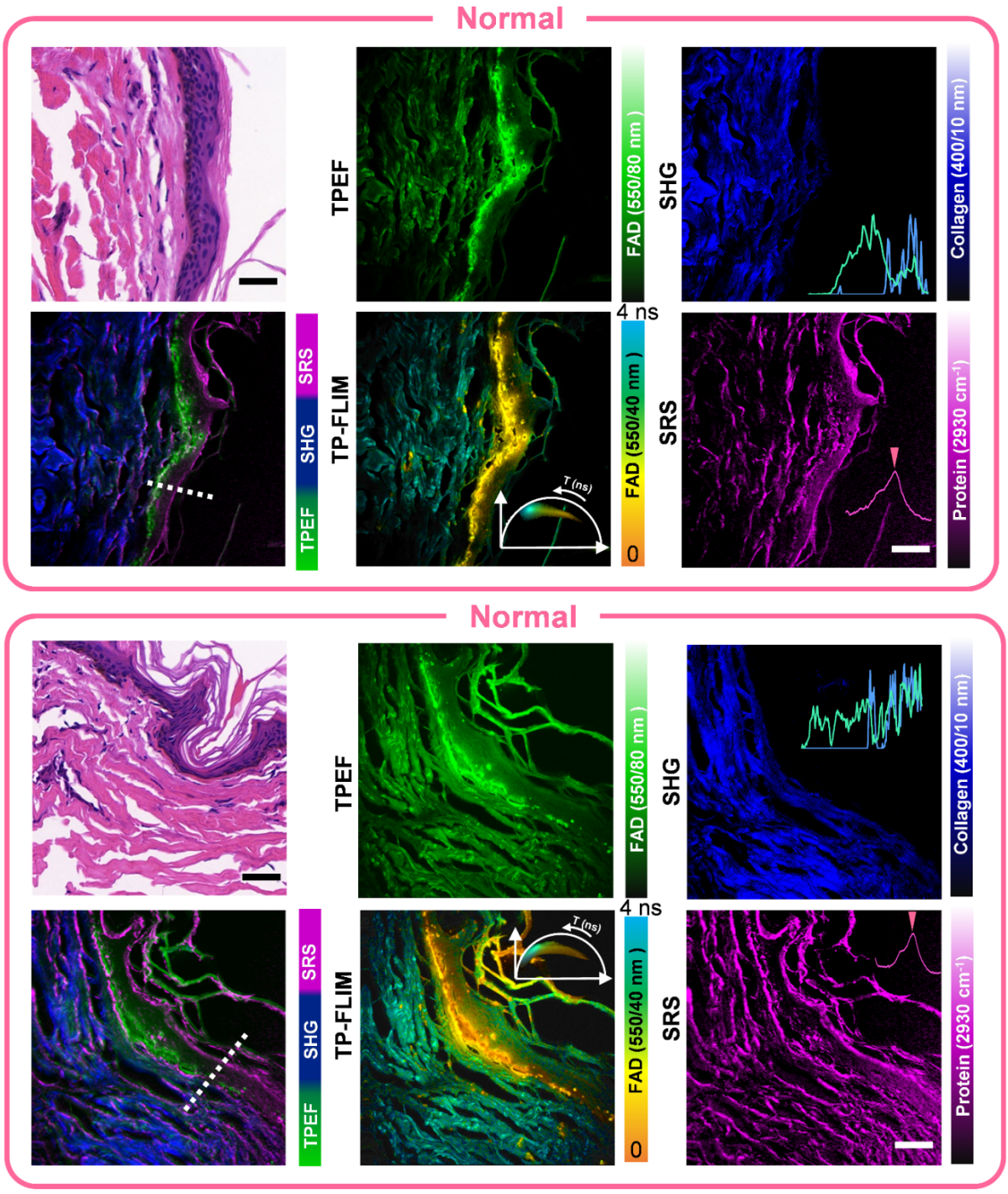
Super-multiple nonlinear optical imaging platform, including TPEF, SHG, SRS and two-photon fluorescence lifetime microscope, have been developed and optimized to capture multiparameter nonlinear optical properties simultaneously for featuring microstructural features and molecular metabolism. This synchronous imaging mechanism accelerates the process of real-time intraoperative evaluation and avoids photobleaching and light damage caused by multiple excitation, which can comprehensively evaluate the pathological characteristics of cancer with its superior spatial, spectral and temporal resolution. However, the excitation conditions of different nonlinear processes (fluorescence and harmonic generation versus coherent Raman scattering) exist significant difference. Specifically, the excitation of TPEF and SHG modalities with a femtosecond laser lead to high photon yield but to poor spectral resolution for CRS. Yet, the picosecond beam as the light source will inevitably lead to the low excitation efficiency of TPEF and SHG, especially the two-photon excited fluorescence lifetime imaging microscope (TP-FLIM) based on single photon counting. In this system, we use a synchronous dual-output femtosecond laser as the excitation light source, in which the vertically polarized beam after the polarization beam splitter is used to excite TPEF and SHG signals, while the horizontally polarized beam is chirped into picosecond beam through the dispersive medium (glass rods) to realize SRS imaging. Notably, this excitation mechanism exists excitation interference and transmission crosstalk, which will lead to low efficiency and inaccuracy of data acquisition. To solve the emission crosstalk, we use the detection of transmission for SRS, and reflection for TPEF and SHG with suitable filter combinations. There are three challenges for excitation crosstalk originated from simultaneous implementation of the three ultrashort pulses. Firstly, the spatial imbalance of multi beam excitation will result in the uneven SRS images and the image ghosting of TPEF and SHG, which will eventually lead to the decline of SRS excitation efficiency and TPEF spatial resolution. The demodulation deviation in SRS imaging is the second challenge. This is because the pump beam in SRS is the wavelength same as the femtosecond pulse used for TPEF (FLIM) excitation, even though it is temporally isolated from the Stokes pulse to avoid interacting with the molecule vibrations. We insert a polarization beam splitter (PBS) into the detection path to isolate the vertically polarized femtosecond pulse by polarization separation before the beam enters the PD for improving the demodulation efficiency and image contrast of SRS. The third challenge is the temporal issue, especially the convolution of fluorescence lifetime in TP-FLIM imaging, which is caused by the staggered excitation of femtosecond pulse and picosecond pulse and eventually lead to the rather inaccurate determination of fluorescence lifetime. Therefore, under the condition of comprehensive consideration, we adjust the τ-delay achieve a time interval between the femtosecond and picosecond pulses. The interval is much larger than the pulse width of picosecond pulses to reduce thermal storage and photobleaching, and less than the time resolution of TP-FLIM to avoid lifetime convolution.



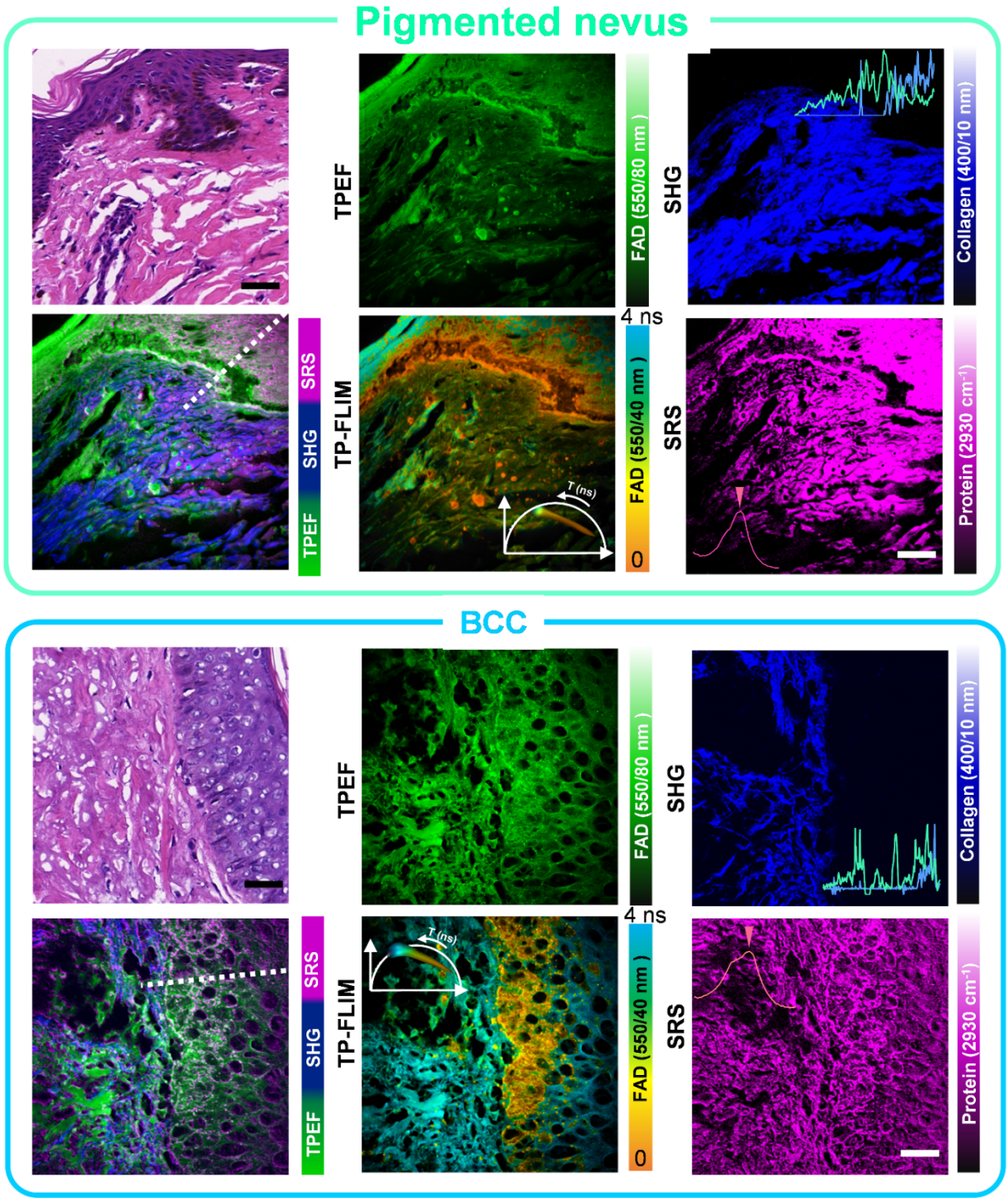
Supplementary Figure 1 Schematic and performance of the multicontrast nonlinear microscopy. CO, condenser; DAQ, data acquisition system; DM, dichroic mirror; EOM, electro-optic modulator; GR, glass rod; λ/2, half-wave plate; OB, objective; PBS, polarizing beam splitter; PD photodiode; PMT, photomultiplier tube; λ/4, quarter-wave plate. Scale bars, 30 μm.



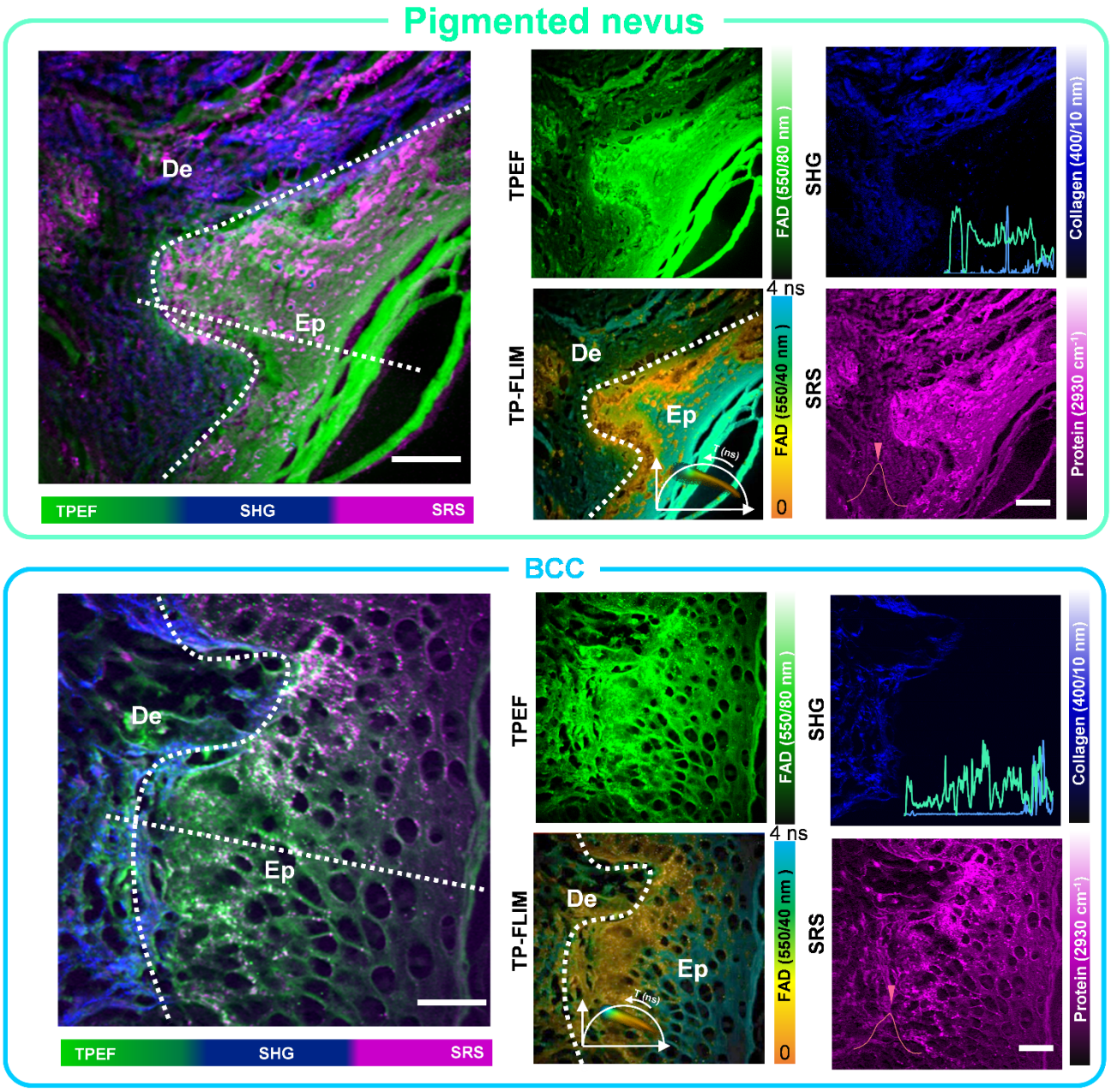
Supplementary Figure 2 Representative examples of the major histological types of skin carcinoma. Yellow arrow: melanocytes; yellow dashed box: lesion area of basal cell carcinoma. Scale bar, 30 μm.



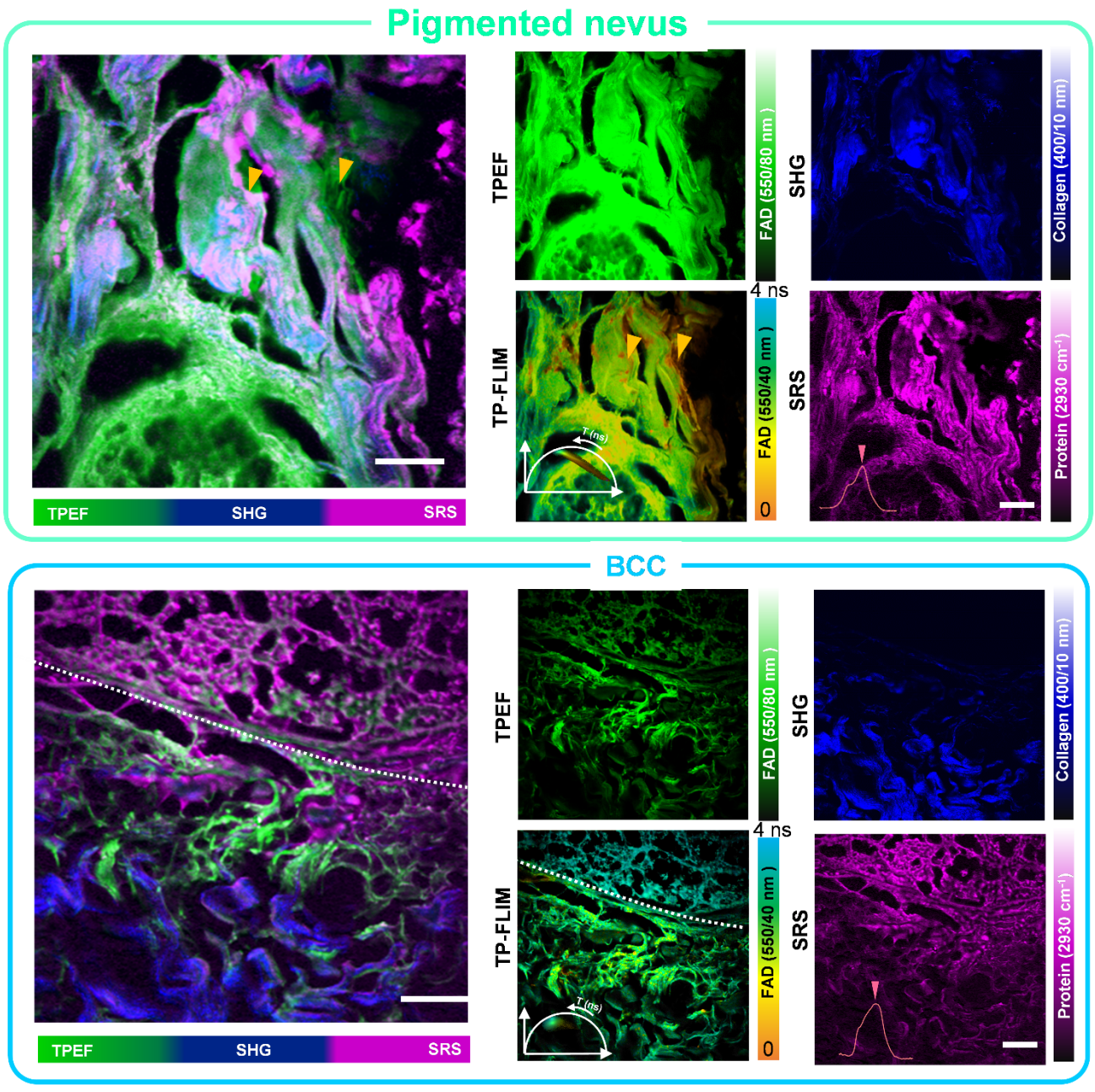
Supplementary Figure 3 Multidimensional nonlinear optical images analysis of normal human skin tissues. Green-TPEF (FAD); blue-SHG (collagen fibers); magenta-SRS (protein). The green and blue curves reveal the epidermis thickness, the magenta curves reveal the overall SRS spectra, and the illustration in TP-FLIM image represent the phasor analysis of endogenous fluorescence (FAD). The color bar in the SRS, SHG, and TPEF images represent the normalized intensity, and those in the FLIM images represent the lifetime range. Scale bar, 30 μm.



Supplementary Figure 4 Multidimensional nonlinear optical images analysis of pigmented nevus and BCC. The illustrations in SHG, SRS and TP-FLIM respectively represent the epidermis thickness, overall SRS spectra and phasor analysis of endogenous fluorescence for revealing the morphological structure and molecular metabolic characteristics of different pathological types of skin tissues. Scale bar, 30 μm.



Supplementary Figure 5 Multidimensional nonlinear optical images analysis of the epidermis in pigmented nevus and BCC. The illustrations in SHG, SRS and TP-FLIM respectively represent the epidermis thickness, overall SRS spectra and phasor analysis of endogenous fluorescence for revealing the morphological structure and molecular metabolic characteristics of the epidermis in different pathological types of skin tissues. Scale bar, 30 μm.



Supplementary Figure 6 Multidimensional nonlinear optical images analysis of the dermis in pigmented nevus and BCC. The illustrations in SRS and TP-FLIM respectively represent the overall SRS spectra and phasor analysis of endogenous fluorescence for revealing the molecular metabolic characteristics of the dermis in different pathological types of skin tissues. Scale bar, 30 μm.

Supplementary Table 1 Specified excitation/detection parameters used in multicontrast nonlinear microscopy.

Nonlinear Optical Imaging	Excitation band (nm)	Pulse shape	Filter detection band (nm)	Target substances
TPEF	800	Femtosecond	550/80	FAD, Elastin
SHG	800	Femtosecond	400/10	Collagen
SRS	Pump:800 Stokes: 1040	Picosecond (linearly chirped)	850/150	Lipid, protein
TP-FLIM	800	Femtosecond /Picosecond	550/40	Bound- and Free FAD