Electronic Supplementary Information (ESI)

S1. Compact nonlinear laser scanning microscope

The optical layout of the microscope as displayed in figure 2 panel A has been designed to fit the requirements of nonlinear SHG/TPEF/CARS microscopy for tissue imaging. Therefore all optical elements in the illumination beam path are optimized for highest NIR transmission. This configuration enables using relatively low power but ultra compact, robust and maintenance free fiber lasers described in the preceding section. In order to realize high optical throughput, the number of optical elements is minimized without affecting the optical performance, i.e. diffraction limited spatial resolution. Since nonlinear imaging methods provide 3D sectioning, no confocal microscope design is required. A NIR anti reflective coated achromatic lens (NIR achromatic lens, 12 mm diameter, 15 mm focal length, Edmund optics, USA) is used to collimate the beam to 6 mm diameter, allowing to fully illuminate the back aperture of the microscope objectives and the galvanometric mirror pair (model 6210HSM40, Camtech, USA). The scanning system is equipped with protected silver coated mirrors of 6 mm diameter providing a surface flatness of λ. Adapting the beam diameter to the size of the objective's back aperture guaranties, that the system's spatial resolution is limited by diffraction of the focusing objective. A scan lens system has been designed to image the centre of the galvanometric mirror pair onto the back aperture of the microscope objective. This scanlens sytem is optimized for minimal chromatic aberrations in the NIR spectral range and to provide a large field of view. Furthermore, the distance between scan lens and objective enables insertion of a short pass dichroic mirror (short pass 750nm, size 24 mm x 36 mm, Edmund optics, USA) and a 400 µm piezo driven stage. The dichroic mirror allows for detection of signals in backward direction and the piezo driven stage provides 400 µm travel along the z axis for fine focusing (PIFOC P-725.4CD, PI, Germany) and automated acquisition of z-stacks. The scan lens system consists of 4 NIR coated achromatic lenses arranged in two pairs in a 4f-configuration: 200 mm – 150mm – 150 mm – 200 mm (NIR achromatic lens, 25mm diameter, Edmund optics, USA). Optimization of the scan lens design was performed using Zemax (Radian Zemax, USA). The microscope objective is mounted to the z-stage. For large area scanning a 20 x 0.4 NA NIR corrected long working distance objective is used (Mitutoyo, Japan). The sample is fixed on a motorized xy-stage providing 75 mm by 50 mm of travel and 1 µm positioning accuracy (ScanPlus, Märzhäuser, Germany). Residual laser light and signals are collimated in forward direction by a NA matched VIS achromatic lens of 25 mm diameter and 30 mm focal length (Thorlabs, USA). For the separation of the nonlinear signals in forward direction two short pass filters are used (SP770 and SP750, Semrock, USA). The forward emitted SHG and CARS signals are splitted by a longpass dichroic mirror (LP605, 24mm x 36mm, Thorlabs, USA), filtered by appropriate bandpass filters (CARS bandpass 650/40 Thorlabs, SHG bandpass 400/40 or 514/10, Thorlabs, USA) and focused onto the PMT detectors by achromatic lenses (diameter 25 mm, 30 mm focal length, Thorlabs, USA). TPEF or alternatively backward scattered CARS/SHG signals are detected by a PMT module in epi-direction. Alternatively also TPEF signals can be detected in forward direction. For detection of CARS signals, PMT modules of enhanced sensitivity in the red spectral range are used (H6780-20, H10721-20 and H9305-03, Hamamatsu, Japan), whereas for detecting SHG and TPEF signals in the visible range VIS sensitive PMTs are used (H6780, Hamamatsu, Japan). A summary of the key components of the instrument is given in table 1. The whole system is schematically depicted in figure 1 panel A, while panels B-E show photographs of parts of the system.

Table S1: List of main components of the compact laser scanning microscope

Model	part	manufacturer
6210HSM40	xy-galvanometric mirror	Cambridge Technology, USA
ScanPlus 75 x 50	xy-tanslation stage	Märzhäuser Wetzlar, Germany
P-725.4CD	400µm Piezo z-stage PIFOC	Physikinstrumente, Germany
	Customized Data acquisition hardware	Pascher instruments, Sweden
H10721-20	8mm head on PMT modules	Hamamatsu; Japan
H6780-20	8mm head on PMT modules	Hamamatsu; Japan
H6780	8mm head on PMT modules	Hamamatsu; Japan
H9305-0	Side on PMT modules	Hamamatsu; Japan
BXFM-F	Manual z-focussing unit	Olympus, Japan
20x Plan Apo NIR	20x NA 0.4 long working distance objective	Mitutoyo, Japan

S2. Data acquisition hardware & graphical user interface

The data acquisition soft- and hardware has been designed for controlling the galvanometric mirrors, the motorized xy microscope translation stage, the piezo z-focussing stage and for synchronized data acquisition of up to 4 channels simultaneously. The data acquisition system consists of two parts: the first part is a small desktop computer for running the graphical user interface for complete instrument control via USB and storage of the image data and the second device is the data acquisition hardware controlling the instrument. Both the data acquisition hardware as well as the graphical user interface were specifically designed for this microscope by T.P., Pascher Instruments AB, Sweden. The menus of the graphical user interface are clearly arranged in order to provide intuitive but complete control of the hardware. A screenshot of the software is displayed in figure 2 panel B. From the user interface the scanning start position, stop position and step length for both directions are sent to the data acquisition hardware control unit. The data acquisition hardware has two major parts. The first part is the scan mirror control unit which regulates the position of the x and y mirrors during scanning and the second part deals with the data readout synchronized with the laser scanner. In the hardware control unit the information about the image size combined with several adjusted electronic delay times is converted such, that the unit performs the full 2D sweep without computer intervention. The hardware control unit contains power supplies for preamplifier and analogue to digital conversion electronics in addition to slots for insertion of controller cards. The first card is used for driving the galvonometric scanner and setting the voltages of the photomultiplier tube modules (PMT), the second card controls the motorized xy stage and the piezo z stage via RS-232 serial ports. Another two cards are used for receiving data from a preamplifier and ADC of two detectors via parallel interface ports. In the following the workflow of performing a 2D scan is described in detail.

The location of each point on the sample is determined by the position of the galvanometric mirrors. During the collection of an image the y direction corresponds to the slow axis which is moved in steps. The fast scan direction is the x axis. In x direction the mirror sweeps from one end position to the other in continuous motion at constant angular speed. The image size is quadratic and can be chosen from 128 x 128 up to 4096 x 4096 pixels. According to the chosen frame size the digitizing electronic collects during the sweep of the fast x-axis the required number of data points, which are equally spaced in time. The first part of the hardware control unit moves the galvanometric mirror pair by automatically performing a full xy scan digitally. The digital position values are converted to voltages by two 16-bit digital to analogue converters (DAC) and the resulting driving voltage signal is sent to the galvanometric scanner. The resolution of the system is sufficient to enable diffraction limited

spatial resolution. 20 voltage steps in the digital position correspond to a beam displacement of 1 µm in the image plane in combination with a 20 x objective. The control unit also provides a trigger signal for each sweep of the X direction mirror to synchronize the data readout with the mirror motion. The second part of the control unit deals with synchronized readout of the detector signals. The readout circuitry is divided into three parts. The first part includes a PMT or diode detector pre amplifier with different computer controllable gain and analogue low pass filters. These settings enable optimisation of the signal to noise ratio for the available absolute signal amplitudes and readout speeds ranging from 1 to 256 µs per pixel. The second component contains a digitizing part consisting of an 18-bit analogue to digital converter (ADC) running at 2 MHz (mega samples per second), which allows averaging a number of readings for each data point. Upon each line triggering event this unit sends the predetermined number of appropriately averaged data points representing a full sweep of the X direction mirror to the data storage card. The storage card as the third component has two memory banks used in ping pong fashion. While the digitizing part files the signal in one storage bank the computer reads out the other one containing the signals of the previous complete line sweep. In principle bidirectional and unidirectional scanning of the sample is possible. For data acquisition the unidirectional scan mode is used, since for bidirectional scans certain delays have to be adjusted depending on image size and the scanning field of view in order to correct for a spatial position offset of the laser beam.
The graphical user interface enables complete instrument control, i.e. setting the PMT voltages, zooming into the image, setting the image parameters, e.g. frame size and the number of pixels, additional filtering, analogue and digital gain as well as point averaging by slow scanning or frame averaging in order to detect weak signals. The options are accessible by drop down lists. An additional menu is popping up for performing a series of images. As indicated in the screenshot shown in figure 2, four options are available: a time series scan for investigating dynamic processes, z-scan for depth resolved imaging of thick samples, xy-scan using the motorized stage for analysing sample areas exceeding the field of view of the scanner and xyz-scans for investigating extended thick specimen. There is no limit on the number of individual images per scan apart from the capacity of the hard disk. Each image of an individual detector is stored as a separate 16 bit TIFF file in addition to a text file containing the settings of the electronics. For adjustment, two fast scan options are implemented. The alignment mode performs a fast scan by reducing the number of lines per image and thus speeding up the image update rate. In the focus mode a fraction of the image is displayed at full resolution for focusing purposes.
The key aspect for adaption of the electronics' hardware of the instrument to nonlinear imaging is a large set of options to optimize the detection of weak signals. These options include low pass filtering of the signal, an additional tenfold analogue gain setting, pixel dwell times up to 256 µs and averaging of a specific number of frames. The most important parameters of the hardware control unit are summarized in table 2.

Table S2: Summary of the parameters of the data acquisition hardware control unit

Resolution	128 x 128 … 4096 x 4096 pixels
Scanning speed	8 fps (128 x 128)… 24 s (4096 x 4096)
Field of view	1,2 mm x 1,2 mm @ 20x*
Detection channels	3 PMTs + 1 photodiode diode
Data depth	16 bit
Pixel dwell time	1 µs…256 µs
ADC	2 MHz