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Supplementary information

Multispectral confocal 3D imaging of intact healthy and tumor tissue using mLSR-3D

In the format provided by the authors and unedited

Supplementary information

Supplementary Methods

Analysis

Segmentation was performed after preprocessing (according to protocol Procedure 2 **Steps 20-25** the mLSR-3D data. We quantified nucleus volume and KI67 positivity using the STAPL-3D segmentation and feature extraction modules as described previously[1]. First, a nucleus mask was created by Difference of Gaussian (DoG) filtering of the DAPI channel (sigmas: 1.5 um and 3.0 um) with Sauvola thresholding (window: [19, 75, 75] voxels, k: 0.2) augmented by a simple intensity threshold of I>0.001 and an absolute minimum threshold of I>0.0007. Then, the mask was segmented into individual nuclei through peak detection (window: [5, 15, 15] voxels; threshold: 1.0 um) in the mask's euclidian distance transform image followed by watershed segmentation. Using the STAPL-3D feature extraction module, the volume and the mean intensity of the 3D-inhomogeneity-corrected KI67 channel were computed for each segment. The segmentation and calculated features were backprojected into the dataset's native space for visual inspection (Figure 6c-d). Segments were filtered by volume >2500 voxels to exclude small segments that do not represent nuclei. After filtering, we had a total of 190,020 nuclei in the high-grade, and 285,153 nuclei in the low-grade glioma dataset. KI67 median intensity was Z-score normalized such that the mean of all the values is 0 and the standard deviation is 1. The threshold for identifying $KI67^+$ cells vs $KI67^-$ cells was set at 2.5.

Clearing Comparison

iDISCO was performed as previously described in *Renier et al, 2014*[2]. In short, PFA-fixated slices of human fetal kidney were washed twice in PBS and incubated in increasing concentrations of MeOH (50%, 80% & 100%, 1 hour each) after which the samples were bleached in 5% H_2O_2, 20% DMSO in MeOH at 4°C overnight. The next day samples were washed twice 1 hour in MeOH, twice 1 hour in 20% DMSO/MeOH and rehydrated in decreasing concentrations (80% MeOH, 50% MeOH, PBS, 1 hour each) and 1 hour in PBS/0.2% (v/v) Triton X-100. The pretreated samples were subsequently incubated overnight in 0.2% (v/v) Triton X-100, 20% (v/v) DMSO and 0.3 M glycine in PBS at 37°C on a slow rocking shaker and blocked for three days in 0.2% (v/v) Triton X-100, 10% (v/v) DMSO and 6% (v/v) FBS in PBS at 37°C on a slow rocking shaker. After blocking the samples were washed for 1 hour in PTwH (0.2% Tween-20 and 10 µg/ml heparin in PBS) and incubated overnight with primary antibodies dilutions 5% DMSO and 3% FBS in PTwH at 37°C. Next day samples were washed thrice in PTwH and incubated overnight with secondary antibody dilutions in 3% FBS in PTwH at 37°C. Next day samples were washed thrice in PTwH and incubated overnight with conjugated antibody and dye dilutions in 3% FBS in PTwH at 37°C. After incubation the samples were washed thrice during the day in PTwH before continuing with dehydration. Samples were incubated overnight in 50 % tetrahydrofuran (THF) in water at RT with mild shaking followed by an increasing concentration of THF (80% THF, 100 % THF twice, 1 hour each). Next the samples were incubated in dichloromethane (DCM) until they sank to the bottom and cleared overnight by refractive index matching with dibenzyl ether (DBE). Cleared samples were mounted in a silicon chamber on a microscope slide and subsequently imaged.

CUBIC was performed as described by Susaki et al. 2014[3]. Briefly, PFA-fixated slices were washed twice in PBS and incubated in Sca*l*eCUBIC-1 (25% wt/v urea, 25% wt/v Quadrol in dH_2O) for 3 days and subsequently washed in PBS. The washed sample was immersed in 20% (w/v) sucrose in PBS and frozen overnight in O.C.T. Compound at -80°C. The next day the frozen sample was thawed and washed with PBS during the day. Primary antibody labelling was done overnight in 0.1% Triton X-100, 0.5% BSA and 0.01% sodium azide in PBS at 37°C with mild shaking. After this, the stained samples were washed trice with 0.1% Triton X-100 in PBS (PBT) during the day and incubated overnight with secondary antibody dilutions at 37°C with mild shaking. Next day, the stained samples were washed trice with 0.1% Triton X-100 in PBS (PBT) during the day and incubated overnight with conjugated antibody and dye dilutions at 37°C with mild shaking. After staining the samples were washed thrice in PBT during the day and immersed in Sca*l*eCUBIC-2 (25% urea, 50% sucrose and 10% triethanolamine in dH_2O) until clear. Cleared samples were mounted in a silicon chamber on a microscope slide and subsequently imaged.

Ce3D was performed as published by Li et al. 2019[4]. In short, PFA-fixated slices of human fetal kidney were washed twice in PBS and blocked 8 hours in 1% normal mouse serum, 1% BSA and 0.3% Triton X-100 in PBS at 37°C with mild shaking. Next the samples were incubated overnight with primary antibody dilutions in blocking buffer at 37°C with mild shaking. After incubation the samples were washed thrice in 0.3% Triton X-100 and 0.5% 1-thioglycerol in PBS during the day and incubated with secondary antibody dilutions in blocking buffer overnight at 37°C with mild shaking. Next day the samples were washed thrice in 0.3% Triton X-100 and 0.5% 1-thioglycerol in PBS during the day and incubated with conjugated antibody and dye dilutions in blocking buffer overnight at 37°C with mild shaking. Stained samples were washed thrice in washing buffer during the day and, after gently dabbing the sample dry with a Kimwipe, incubated in increasing gradient of Ce3D clearing solution (40% N-methylacetamide, 86% Histodenz, 0.1% Triton X-100 and 0.5% 1-thioglycerol; 33%, 66%, for 2 hours, in PBS) and finally submerged in 100% Ce3D overnight. Cleared samples were mounted in a silicon chamber on a microscope slide and subsequently imaged.

Each condition was imaged on the same day with the same acquisition settings and laser powers. Subsequent quantification was done by plotting the mean intensity of the top 10% highest intensity pixels per *z*-step for every channel in each condition. Channels without specific signal were removed from quantification.

Two-photon microscopy

Two-photon microscopy (Extended Data Fig. 4) was performed on the same Zeiss LSM880 with 32-channel spectral detector as used in the rest of the method. The objective was a 32× NA 0.85, water immersion with a working distance of 1.1mm. Spectral signature references were generated with the lambda-mode specifically for this two-photon setup and used for online fingerprinting to apply linear unmixing of the overlapping emission spectra into separate channels during acquisition.

References

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4. Li, W., Germain, R. N. & Gerner, M. Y. High-dimensional cell-level analysis of tissues with Ce3D multiplex volume imaging. *Nat Protoc* 14, 1708–1733 (2019).

Method comparison at 25× for 250 µm stack

Method	**mLSR-3D**	**Compensation correction (Gerner 2012)**	**Sequential scan (Coutu 2018)**	**Sequential lambda scan**
Microscope	Zeiss LSM880	Zeiss LSM710 / Leica SP5	Leica SP5	Leica SP8
Number of colors	8	7	8	8
Number of scans needed	1	2	4	32
Scan time Z-Stack	10 min	22 min	45 min	431 min
Raw data size	NA*	3 GB	3.5 GB	14 GB
Processed data size	3.25 GB	3 GB	3.5 GB	3.5 GB

*Data not stored

Supplementary Table 1 | Method comparison for single *z*-stack. Comparison of number of scans needed, absolute scan time and raw and processed data size for mLSR-3D, Gerner et al 2012[47], Coutu et al 2018[32] and sequential lambda-scanning when using similar acquisition parameters; Bit depth 16, pixel dwell 2 µs, voxel size 0.33 x 0.33 x 1.2 µm.

Typical acquisition settings			
Objective	10×	25×	40×
Bit depth	16	16	16
Pixel dwell (µs)	2	2	2
Frame size (pixel)	2048 × 2048	1024 × 1024	1024 × 1024
Step size (µm)	3,2	1,2	0,47
Voxel size (µm)	0.42 × 0.42 × 3.2	0.33 × 0.33 × 1.2	0.21 × 0.21 × 0.47
Working distance (µm)	2000	570	280

Supplementary Table 2 | Typical acquisition settings and working distance for 3 magnifications. Zeiss Plan-Apochromat 10x/0.45 M2; LD LCI Plan-Apochromat 25x/0.8 Imm Corr DIC M27; C-Apochromat 40x/1.2 W AutoCorr M27.