Multivariate Analysis of 3D ToF-SIMS Images: Method Validation and Application to Cultured Neuronal Networks

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Supplementary Materials and Methods (Cell Sample)

Cell Substrate Preparation

The 22 x 22 mm^2 glass coverslips (631-1570 - VWR) were solvent cleaned with methanol (HPLC), acetone (LRG) and isopropanol (HPLC). All solvents were purchased from Fisher Scientific and used as obtained. After each solvent rinse, the coverslips were blow-dried with compressed air. Then, adhesive polyester microscope slide-grids with 1 mm line spacing (Z688533 - Sigma) were cut to fit the glass coverslips and stuck onto the glass. The coverslips were then placed in a vacuum oven (Thermo Scientific Vacutherm 6025) at 60 °C and 50 mbar for 1 h and rinsed with isopropanol (HPLC) again before being stored in a vacuum desiccator. Before the cell plating, the coverslips are aseptically coated with 0.01 % (w/v) poly-L-lysine (P4707 - Sigma) in water. After 40 minutes, the poly-L-lysine solution is removed by aspiration and thoroughly rinsed with sterile tissue culture grade water (W3500 - Sigma). The coverslips were allowed to dry for at least 12 hours before introducing cells and medium.

Cell Culture

Low-density cultures of disassociated embryonic rat cortical neurons were prepared and cultured in serum-free media on the poly-L-lysine coated glass coverslips. All experiments involving animals were performed according to the UK Home Office regulations in accordance with the Animals (Scientific Procedures) Act 1986 and were approved by the University of Nottingham's animal welfare committee. Cortices were dissected from rat embryos at day E18 of gestation and incubated in 2 ml of HEPES-buffered Hanks' balanced salt solution (HBSS), containing 1 % (w/v) trypsin (T1426 - Sigma) and 0.005 % (w/v) DNAse (D5025 - Sigma) for 20 min at 37 °C. After that, 0.05% (w/v) trypsin inhibitor (T9003 - Sigma) was added to de-activate the trypsin. The cortices were then rinsed with 1.5 mL of Neurobasal medium and gently triturated using a fire-polished glass Pasteur pipette in the presence of 0.005 % (w/v) DNAse. The resulting cell suspension was then centrifuged and the pellet re-suspended in Neurobasal media containing B27 supplement (17504-044 - Invitrogen), 0.5 mM L-glutamine (G7513 - Sigma) and 1 % (v/v) of Penicillin-Streptomycin (P0781 - Sigma). The prepared coverslips were placed in 6-well tissue culture plates and plated with a total of 150,000 disassociated cells per coverslip. After incubating for 30 min at 37 °C to allow cell adhesion to occur, the wells were flooded to a total volume of 2 mL of Neurobasal/B27 media. After 24 h, the media was replaced with fresh media and the cultures were maintained at 37 °C in a humidified 5 % CO_2 atmosphere for the next 9 days.

Cell Preparation

The total osmolality of the cell culture media was first determined with a cryoscopic osmometer (Osmomat 030) and equalled 237.3 ± 4.7 mOsm/L (n = 6). Before being flash-frozen by plunging the coverslips into 20-30 mL of liquid ethane for 20 s, the cell covered coverslips were dipped three times with a pair of tweezers in a 119 mM ammonium formate (516961 - Aldrich) solution that matched the measured osmolality of the cell culture media. The ethane was liquefied by letting a stream of ethane gas condensate into a 50 mL Falcon tube held in a bath of liquid nitrogen. The samples were stored in liquid nitrogen until the freeze-drying process. The cold samples were transferred to the freeze-dryer (VirTis Benchtop Freeze Dryer) in falcon tubes and the chamber was immediately pumped down to c. 10 Pa. When the freeze-drying process was complete, the samples were stored in a vacuum desiccator. Well-preserved cells were identified by comparing light microscopy images from before and after the cell preparation.

Optical Microscopy

Optical microscopy images were captured on a Nikon Eclipse Ti inverted microscope with a 20x/0.50 objective and an ORCA-Flash4.0 (C11440 - Hamamatsu) digital CMOS camera (2048 x 2048, 6.5 µm pixels). The resulting images are analysed and processed using ImageJ.

Interferometry

In order to assess the samples' surface topography, a 3D mapping was achieved using a vertical scanning interferometer (Fogale Nanotech Photomap 3D; 763 x 573, 9.4 µm pixels) with a 5x/0.12 objective. The total roughness R_t and root-mean-square roughness R_q are calculated from the data using Matlab (Release 2013a, The MathWorks, Inc., Natick, Massachusetts, United States). The total roughness R_t is calculated as $\max(z_i) - \min(z_i)$ and the root-mean-square roughness R_q is calculated as $\mathrm{sqrt}(\mathrm{mean}(z_i^2))$ with z_i the vertical distance from the mean line to the i^{th} data point.

ToF-SIMS

The ToF-SIMS analyses were executed using a ION-TOF TOF-SIMS IV instrument (Münster, Germany), equipped with a Bi liquid metal ion gun (LMIG) and Ar gas cluster ion beam (GCIB). The primary ion beam is directed at the sample under an angle of 45 ° in relation to the normal and has a beam spot of 1-2 µm in the high-current bunched mode. 25 keV Bi_3^+ primary ions were used in all measurements. Charging of the sample is compensated with the low-energetic electrons of the flood gun. 20 keV Ar_{5000}^+ ion clusters were used for sputtering. Ion images were recorded in the high current bunched mode, which allows for higher mass resolution. 250 x 250 μm^2 raster scans with 256 x 256 pixels and 5 frames per scan (1 shot per pixel) were analysed. With a target current of 0.3 pA in AC mode, this resulted in a ion dose of 1.02 10^{13} primary ions cm^{-2}, which approximates the static limit. The mass resolution was on average 1810 (FWHM) at m/z 15. The 20 keV Ar_{5000}^+ GCIB sputtered a region of 500 x 500 μm^2 with a target current of 1.2 nA for 5 s between the 160 scans. The positive ion mass spectra were calibrated with m/z 1 (H^+), 15 (CH_3^+), 29 ($C_2H_5^+$), 43 ($C_3H_7^+$) and 57 ($C_4H_9^+$).

Data-processing

A peak search was performed to locate relevant mass peaks with a minimum of 15,000 counts, a minimum of 3.0 SNR and a maximum of 0.8 background and secondary ion images were reconstructed from the raw data files with the commercial ION-TOF software (Surfacelab 6). These values allowed all major peaks in the spectra to be selected and were determined empirically for these specific data sets. Images for the integrated peaks were then exported in an ASCII file format for further data processing in Matlab (Release 2013a, The MathWorks, Inc., Natick, Massachusetts, United States). All further data processing was performed using in-house generated Matlab scripts. All calculations were performed on a 64-bit Windows 10 platform with 8GB of RAM, using an Intel Core i3, 1.8GHz processor.

Supplementary Figures

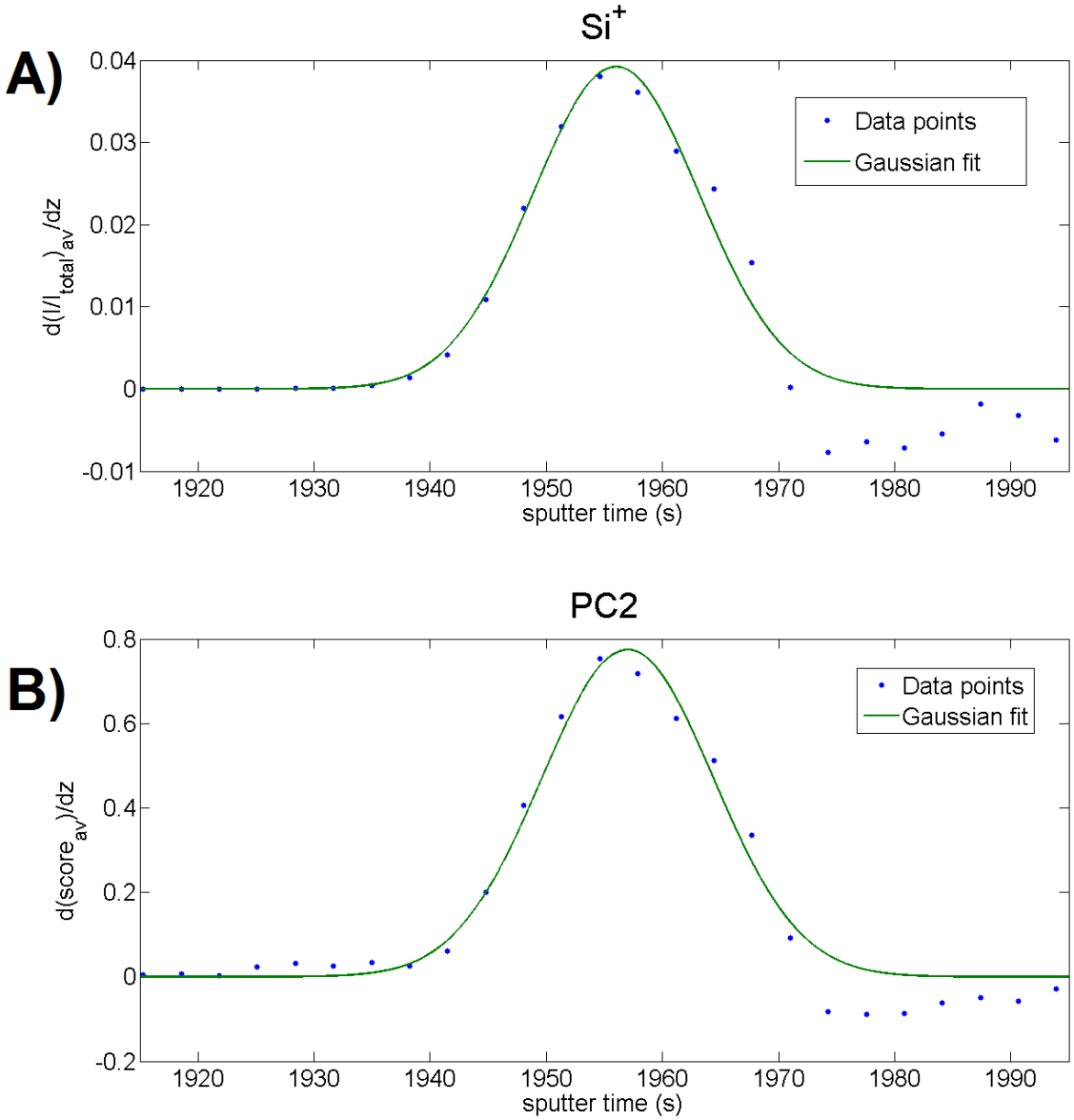

Figure I. Z-calibration for the PS-PVP multilayer sample using the Si wafer interface. A) Gaussian curve fit (a exp -((x-b)/c)^2 with a = 0.03921, b = 1956 and c = 10.13) to the gradient of the average Si^+ intensity in the z-direction. This is used to scale the ion images. N.B. only the planes in the vicinity of the interface are shown. B) Fitting a Gaussian curve (a exp -((x-b)/c)^2 with a = 0.7744, b = 1957 and c = 10.48) to the gradient of the average PC2 scores in the z-direction. This is used to scale the scores images. N.B. only the planes in the vicinity of the interface are shown.

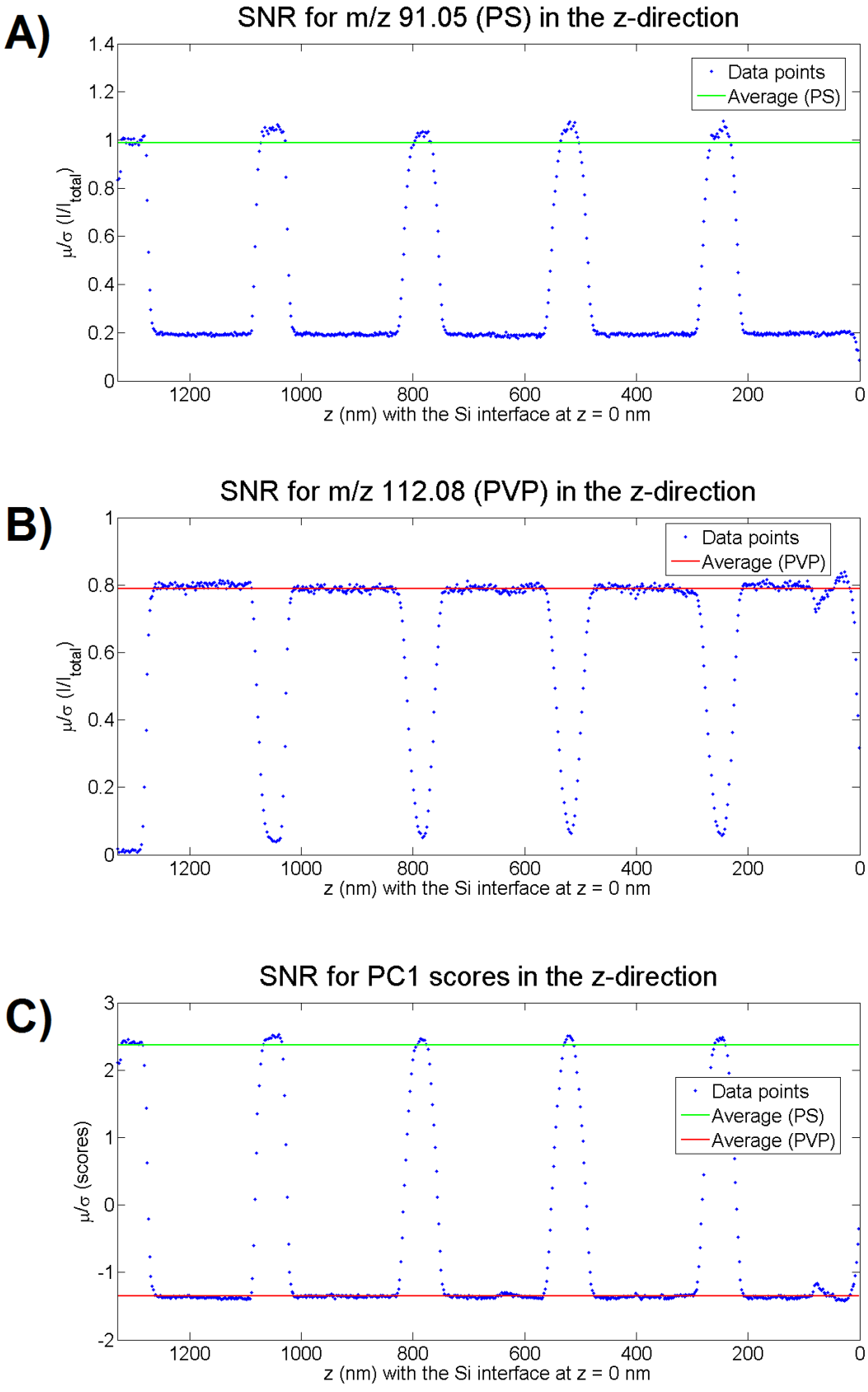

Figure II. Calculation of the SNR for the PS-PVP multilayer sample ion and scores images. The SNR is calculated as μ_{sig}/σ_{sig} to allow direct comparison between ion images and scores images. A) The SNR for m/z = 91.05 (PS) equals 0.99. B) The SNR for m/z = 112.08 (PVP) equals 0.79. C) The SNR for the positive scores (PS) of PC1 equals 2.4 and the SNR for the negative scores (PVP) of PC1 equals 1.35.

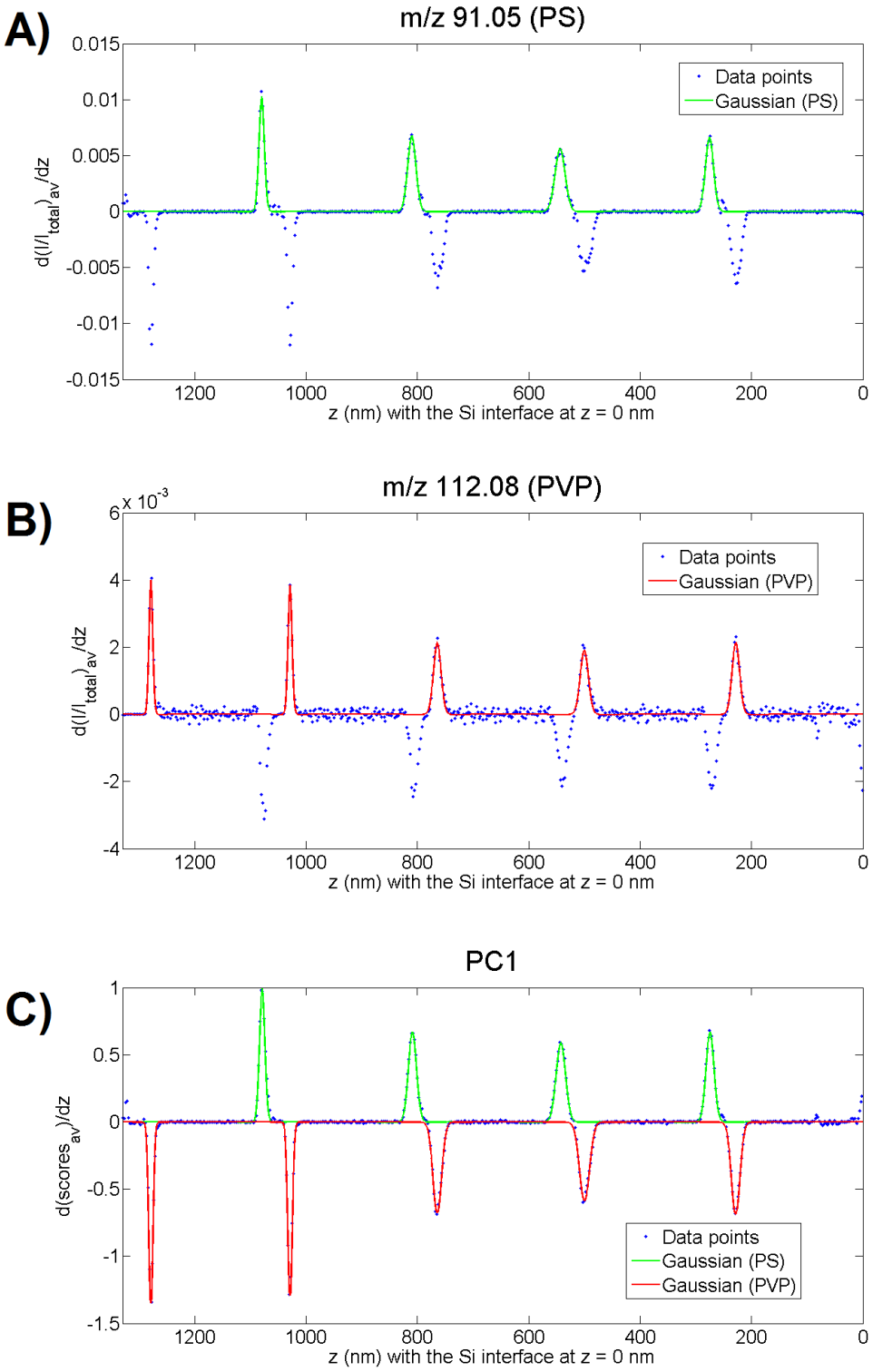

Figure III. Calculation of the depth resolutions Δz for the PS-PVP interfaces in the ion and scores images. The depth resolutions Δz = 2σ are calculated by fitting Gaussian curves (a_i exp -((x-b_i)/c_i)^2) to allow direct comparison between ion images and scores images. A) Fitting Gaussian curves to the gradient of the average m/z = 91.05 (PS) intensity in the z-direction. B) Fitting Gaussian curves to the gradient of the average m/z = 112.08 (PVP) intensity in the z-direction. C) Fitting Gaussian curves to the gradient of the average scores for PC1 in the z-direction. The parameters of the Gaussian functions can be found in Supplementary Table I.

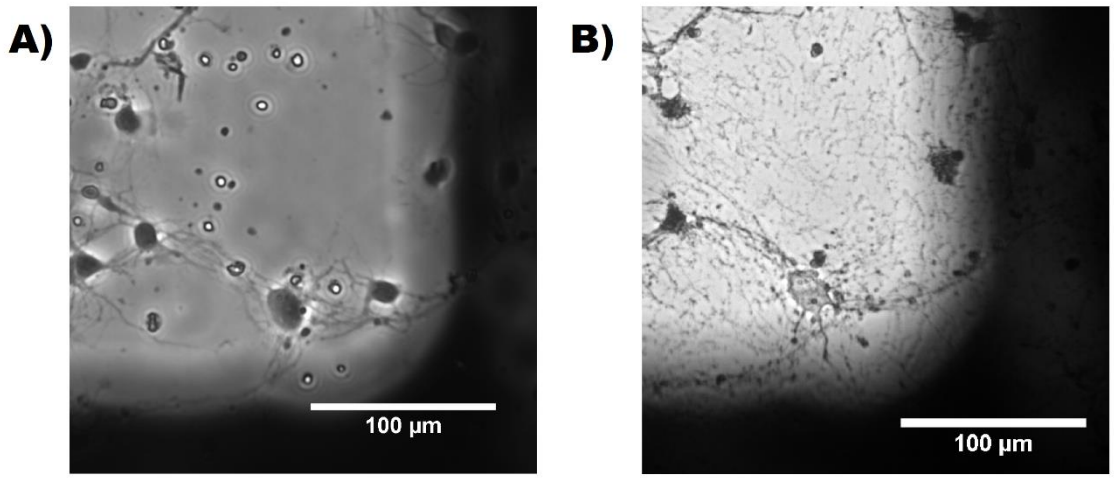

Figure IV. Optical microscopy of the part of the neuronal network investigated with TOF-SIMS. A) Differential interference contrast (DIC) microscopy image (20x) before freeze-drying (7 days *in vitro*). B) Bright-field microscopy image (20x) after freeze-drying. Several neurons (grey ovals) can be seen as well as their axons and dendrites. The right and bottom black edges on the images originate from the grid fixed to the back of the microscope slide, which were used to locate cells during interferometry and TOF-SIMS measurements.

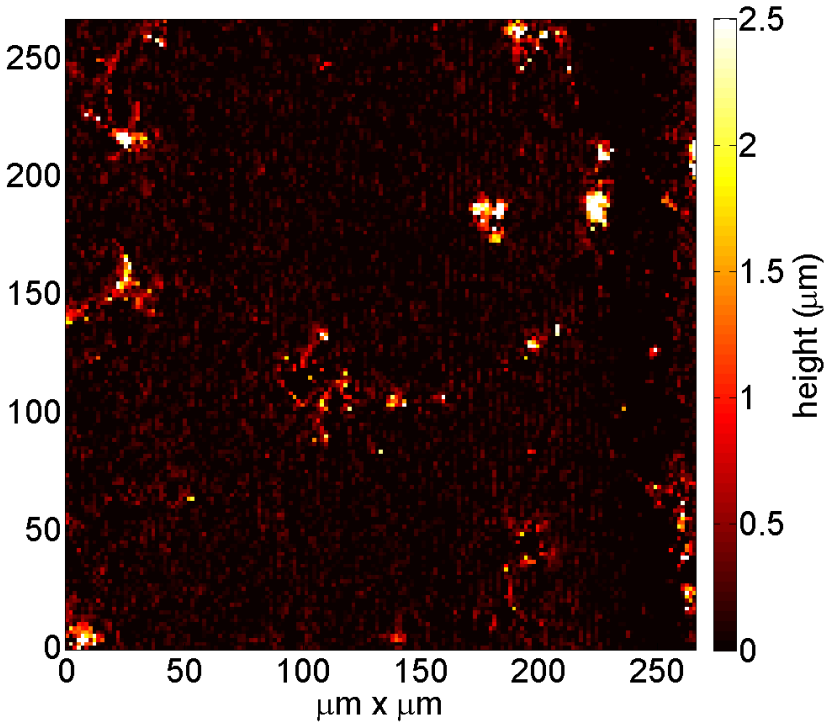

Figure V. White light interferometry heightmap (R_t = 9.6 µm, R_q = 0.36 µm). The maximum height of the features is approximately 2.5 µm.

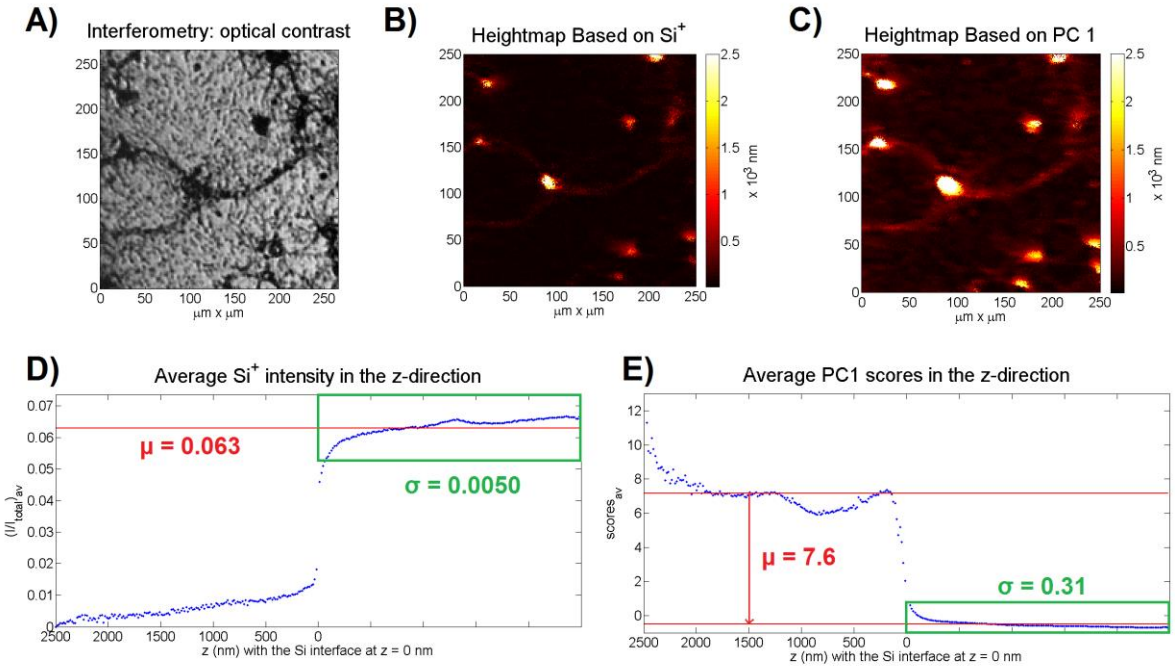

Figure VI. Illustration of the improved z-offset correction when using a principal component instead of a single ion. A) Optical image obtained from the interferometer. B) Heightmap based on the ion intensity of Si^+. The interface is defined as $I_{normalised}$ = 0.05 and taken as a reference for the substrate plane. Scaling is performed using a maximum height of 2.5 µm (based on interferometry data) and assuming a constant sputter rate. C) Heightmap based on the scores for PC1 (48.8% variance explained). In this case, the interface is defined as scores = 0 and taken as a reference for the substrate plane. This heightmap has more features in common with the optical image. D) Calculation of the SNR for the average Si^+ intensity in the z-direction. The SNR equals 13. E) Calculation of the SNR for the average PC1 scores in the z-direction. The SNR equals 25, which is 1.9 times that of the average Si^+ intensity. As a result, a heightmap that resembles the optical image more closely is obtained.

Supplementary Tables

Table I. Parameters of the Gaussian curves (a_i exp -(($x-b_i$)/c_i)^2) fitted to the gradient of the average m/z = 91.05 (PS) intensity, m/z = 112.08 (PVP) intensity and scores for PC1 in the z-direction, seen in Supplementary Figure III, to allow direct comparison between ion images and scores images. The depth resolutions Δz are calculated as 2σ and there is no significant difference between those calculated with the ion images and those calculated with the scores as shown by a pairwise t-test (P = 0.31).

Interface	m/z 91.05 (PS) or m/z 112.08 (PVP)				PC1 scores			
	a_i	b_i (nm)	c_i (nm^2)	Δz (nm)	a_i	b_i (nm)	c_i (nm^2)	Δz (nm)
1 (PVP)	0.003996	1279	5.083	3.19	-1.346	1279	5.132	3.20
2 (PS)	0.010280	1080	7.087	3.76	0.9903	1079	7.181	3.79
3 (PVP)	0.003839	1029	5.400	3.29	-1.290	1029	5.540	3.33
4 (PS)	0.006753	810.3	10.90	4.67	0.6732	809.1	10.56	4.60
5 (PVP)	0.002134	764.6	9.882	4.45	-0.6745	764.6	10.69	4.62
6 (PS)	0.005647	544.0	13.08	5.11	0.5918	542.6	12.20	4.94
7(PVP)	0.001923	500.5	10.65	4.62	-0.5838	500.1	12.38	4.98
8 (PS)	0.006661	275.6	10.58	4.60	0.6717	274.6	10.48	4.58
9 (PVP)	0.002146	228.3	9.854	4.44	-0.6844	228.6	10.58	4.60

Table II. Processing times and memory usage of the PCA performed on the validation data set. All calculations were performed on a 64-bit Windows 10 platform with 8GB of RAM, using an Intel Core i3, 1.8GHz processor.

Process	Time (s)	Input data (Mb)	Output data (Mb)	Peak memory (Kb)
Training set formation	44.3	1386.3	1283.8	1255792
Eigendecomposition	8.5	1283.9	0.012432	1256176
Scores formation	1667.4	2670.0	489.58	1256300