Supplementary Material for: Ion microprobe determination of ex vivo trace element molarities: the LINAEUS code suite

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Abstract
This Supplementary Material document collects technical data and information about the main paper which is useful as a Users reference but out of place in the main text.

Keywords: Trace element analysis, LINEAUS, Particle Induced X-ray Emission (PIXE), Elastic Backscattering Spectrometry (EBS), Off-Axis Scanning Transmission Ion Microscopy (OA-STIM), Dynamic Analysis

1 Introduction

LINAEUS[1] (Linear Analysis of Elements using Spectroscopies) is a code suite that supports quantitative analysis of lesser- and trace-elements using Particle Induced X-ray Emission (PIXE). LINAEUS determines the major matrix elements (C, H, N and O) and section thickness from simultaneous Elastic Backscattering Spectroscopy (EBS) and Off-axis Scanning Transmission Ion Microscopy measurements. The LINAEUS code suite is multi-platform and can be run under Mac-OS, Linux, Windows (10 and 11). The program is undergoing continuous development and changes and extensions will be included in the text from time to time. Much of the formulas have been presented in other work [11, 13, 12, 14, 8, 9] and are collected here for completeness. The notation and terminology follows that used in standard texts on ion beam analysis e.g. [7, 2]. In this Supplementary material various technical details concerning LINAEUS are collected. Figure 1 presents the file structure.

2 Molarity formula

The molar concentration (molarity) is a physiologically significant quantity that is the number of moles per dm^3 (1 litre) of volume. The molarity is thus a quantity that is a function of two fundamental SI units [1].

GeoPIXE and other PIXE analysis codes typically provides the concentration of element in terms of the dry mass ratio m_i/m_m in units of $\mu g/g$. Here, m_i is the mass of the lesser- / trace-element i and m_m is the mass of the matrix. Taking α to be the fraction of tissue covered pixels that contribute to PIXE signal for element i, the number of moles in a region of pixels is,

$$n_i = \frac{1}{\alpha}\left(\frac{m_i}{m_m}\right)\frac{m_m}{A_i} \tag{1}$$

Where, A_i is the atomic mass in g. The thickness t is determined from the energy shift of the H-surface edge (Fig. 5 (b) in the paper) and corresponds to the thickness of tissue pixels. Then if ρ is the dry tissue matrix density,

$$m_m = t\rho \tag{2}$$

is the dry tissue matrix mass per unit area. The volume of the tissue in the frozen tissue section of thickness T per unit area is,

$$V = \frac{T}{\alpha}. \tag{3}$$

Then because the α cancels-out, the molarity of element i is,

$$x_i = \frac{n_i}{V} = \left(\frac{m_i}{m_m}\right)\frac{\rho t}{A_i T}. \tag{4}$$

[1] Carolus Linnaeus is the latin form of Carl von Linné, biologist, natural scientist, medical doctor and rektor magnificus of Uppsala University 1750-1752.

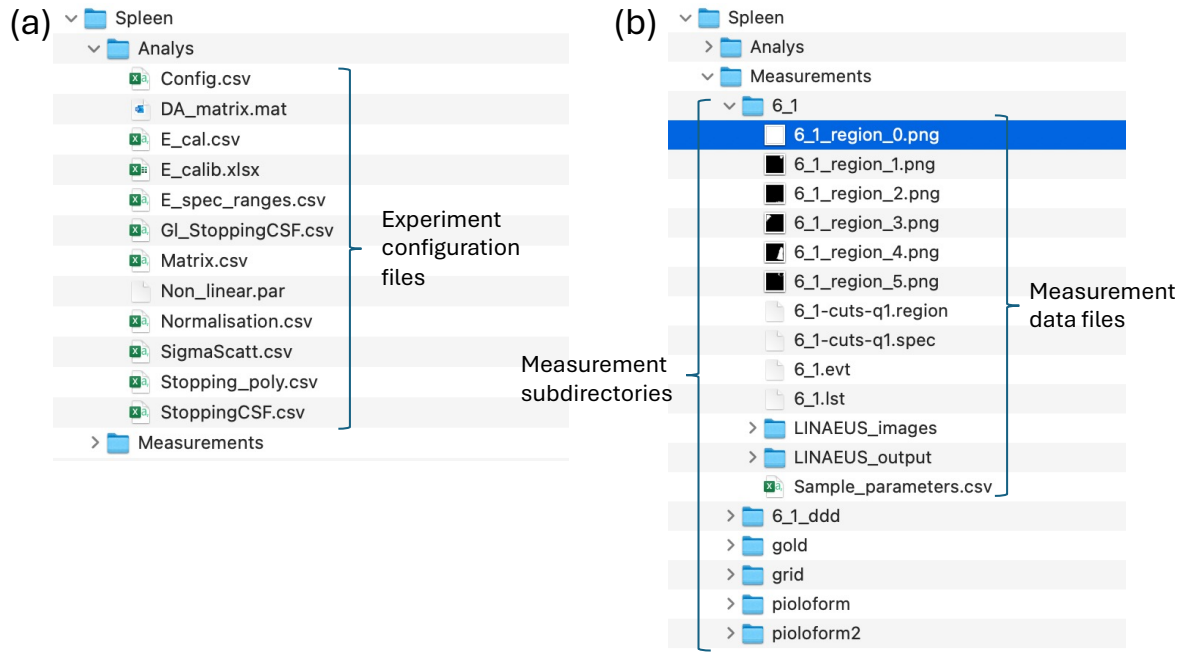

Figure 1: LINAEUS file structure for the experiment name "Spleen". (a) Analys subdirectory expanded to show the experiment configuration files. (b) Measurement subdirectory expanded to show the subdirectories for each measurement with the measurement 6_1 expanded to show the data files. The LINAEUS_images and LINAEUS_output subdirectories hold the .png image files and .csv format Edge height and atomic composition data produced by LINAEUS.

x_i represents the molarity in the tissue without any contribution from the void volumes. It should be considered that α can influence the molarity if it enters into the determination of m_m and hence t. Although ρ enters into the Equation above, it cancels out with the ρ used in the determination of (m_i/m_m) in GeoPIXE. Depending on the units used a constant coefficent may need ot be included to expess the molarity as moles per dm^3.

3 The LINAEUS code suite

The LINAEUS code is written in Python 3.13 and multi-platform and runs under Microsoft Windows 10/11, Mac-OS and LINUX. TKinter is implemented differently on different operating system platforms. To keep the code short, a common GUI coding is used for the different operating system platforms, which unfortunately in some cases gives the GUI a rough and unpolished appearance. The LINAEUS GUI is organised as a series of tabs that lead the user through the analysis procedure by progression from one tab to the next. The program is structured to allow easy extension of functionality by addition of additional code that can be activated by means of the GUI.

Based on the authors' experience of ion beam analysis, LINAEUS is designed to exploit the usual situation in ion beam analysis were in one experiment-session, a number of different measurements are carried out on one, or more, samples (including calibration samples). Therefore the data structure is organised so that all files for an experiment are stored in a single Experiment directory. This is structured into subdirectories shown in Figure 1 The configuration, energy calibration, cross section and stopping data do not usually change between measurements and are stored in the common Analys sub directory. Communication between the different parts in LINAEUS and numerical input/output to/form GeoPIXE is by means of .csv format files. The configuration data files can be edited within LINAEUS, or using a standard spreadsheet program.

Figure 2: Screen-shot tab for setting up the experiment configuration parameters.

3.1 LINAEUS GUI tabs

There are four GUI tabs implemented in LINAEUS that are intended to lead the user though the different steps in the data analysis. These correspond to:

1. Setting up of the configuration data for the entire experiment.
2. Setting up the measurement specific data.
3. Definition of the prototype EBS spectrum by non-linear regression.
4. Dynamic Analysis to obtain the major element composition in each tissue region.

3.2 The Experiment set-up tab

Figure 2 shows the LINAEUS tab for setting-up the experiment configuration data. The setting-up is done by modifying a group of human readable .csv format files contained in the Analys subdirectory. Clickable buttons allow different parameters in the experiment configuration files to be edited. The window for setting up the measurement parameters is shown in Figure 3.

The first step is to select the "Experiment set-up" directory where all the data pertaining to the Experiment in question is collected. This is necessary because the procedures and programs that make up LINAEUS look up file paths to the Experiment directory to collect configuration data. The configuration files pertaining to the experiment are stored in the Analys subdirectory and have predetermined names (Figure 3(a)). (Similarly the configuration files pertaining to a given measurement are stored in a corresponding subdirectory in the Measurements subdirectory (Figure 3 (b)). The configuration .csv files are human readable and can be edited using standard spreadsheet or text-editor programs.

When starting analysis of a new Experiment, the Analys subdirectory can be copied from another experiment in order to provide a initial set of configuration files. This is convenient since, often the configuration is unchanged between different experiments.)

Edit Experiment configuration button This opens a window (Figure 2) that allows the basic experiment configuration to be set-up. The initial step is to select the Analys directory.

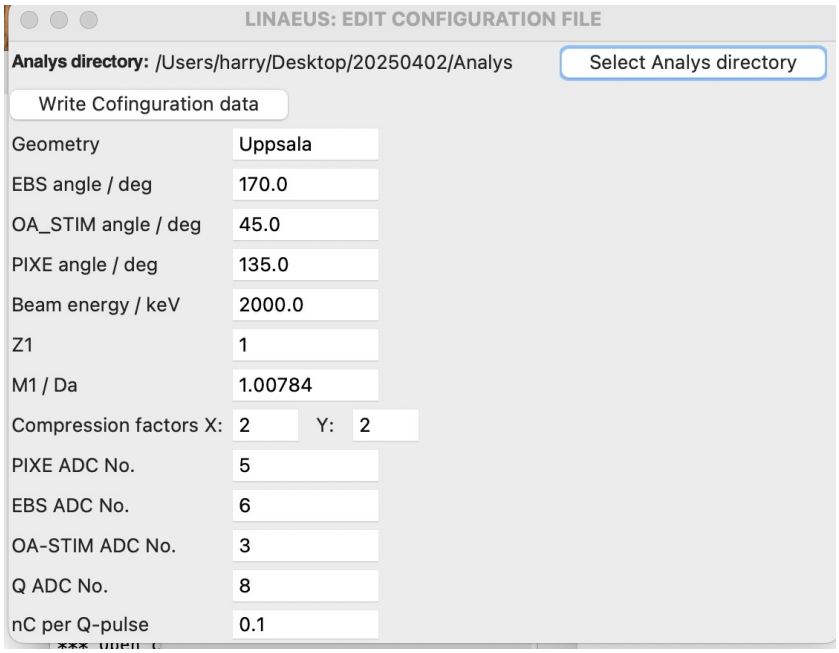

Figure 3: Screenshot of window for setting the experiment parameters.

Read- and Write configuration data buttons. These allow the configuration data to be read from an existing file and new data written to a file.

Editable fields These allow different parameters to be edited. Note that these are handled and treated as string values. Thse are converted to float and integer quantities as needed by the individual codes in LINAEUS.

Edit energy calibration button The window is shown in Figure 4. This window provides a way to manually enter the energy calibrations for an experiment. A more straightforward graphical way to enter the energy calibrations is to use the Data Visualizer described below. The calibration is a straight-line form; $E\,(\mathrm{keV}) = A\,(\mathrm{keV/ch.}) \times x + B\,(\mathrm{keV})$, where x is the channel number. paragraphEdit OA-STIM and PIXE spectrum ranges button This facilitates manually entering the energy channel intervals for the OA-STIM and PIXE energy spectra. The window is similar to the Edit Experiment configuration described above. A more straightforward graphical way to enter the energy calibrations is to use the Data Visualizer presented below. Note that the EBS energy channel number intervals used in the Dynamic Analysis and others are set in the non-linear fitting procedure.

Edit reference button The reference sample data window is shown in Figure 5. Again this is similar to the Edit Experiment configuration described above. This is used to select and manually enter the yields under the H, C and O peaks in the reference sample, the position of the H surface-edge. The channel ranges and yield parameters for the OA-STIM, EBS and PIXE can be obtained graphically using the Data Visualizer and entered into the energy calibration file.

In addition the C,H,N and O atomic compositions can be set for the reference standard (usually Pioloform) and the prototype tissue composition used a zero-order approximation in iteration of the C, H, N and O composition in the Dynamic Analysis step.

Edit EBS scattering cross sections button This window (Figure 6) is used to edit the EBS cross-sections for H impinging on ^{12}C, ^{14}N and ^{16}O. It is only used where no N containing standards exist. The cross sections can be obtained from the SigmaCalc website, or original literature.

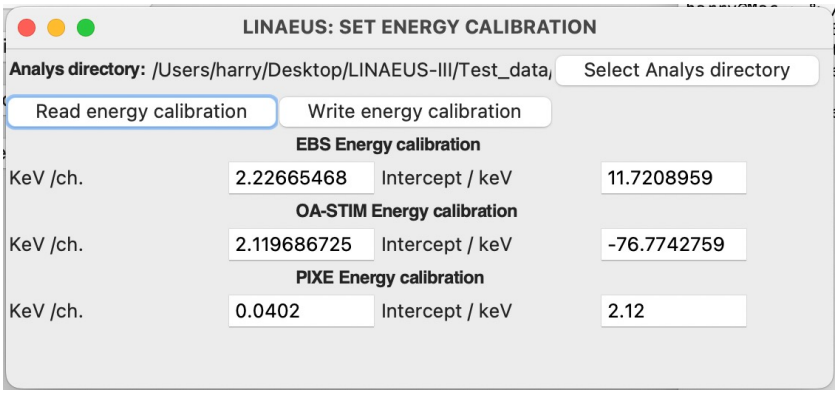

Figure 4: Screenshot of the window for setting the energy calibration parameters.

3.3 The Measurement configuration tab

The purpose of this tab is to configure the steering file for the Dynamic Analysis step for each measurement and also extract calibration data. It also facilitates conversion of .lst format data from FastCom MP3 format .evt. It works on the measurement directory which must be set as the first step. Figure 7 shows the Measurement configuration tab.

Data Visualiser button A useful tool is the "Data Visualiser" which sorts the raw data and visualises it as an energy spectrum and a map is signal yield for the EBS, OA-STIM and PIXE signals. The Data Visualizer which is a separate program that opens in a separate window seen in Figure 8. In addition to displaying the data the Data Visualiser performs a large number of functions such as getting energy calibration data and writing this to the data files in the Analys subdirectory. It is an alternative way to enter the calibration and normalisation data for the experiment. If not automatically set by LINAEUS, the Experiment and Measurement directories have to be selected when the window opens.

OA-STIM, EBS and PIXE buttons When a OA-STIM, EBS or PIXE button is selected, a sort is performed of the measurement .evt file and the resulting the energy spectrum and map of the corresponding signal yield are displayed. By default, the entire energy range and image area is displayed. By selecting smaller energy ranges with the cursor and re-selecting the OA-STIM, EBS or PIXE button the spectrum and map is displayed for this region. In this way, rough maps of the EBS, OA-STIM and PIXE signals for one element can be displayed.

Smoothing button To facilitate checking for realistic peak- and edge-positions when the energy spectrum has significant statistical noise a smoothing function is provided. This uses a Savitsky Golay filter [10] with window length = 9 samples and polynomial order = 3 . This filter was chosen because it has the property that it smooths noisy spectra while preserving maxima and minima and edge positions.

Select region button A region file can be selected using the GeoPIXE (.csv format) or as a black/white mask (.png) format. The clicking on the OASTIM, EBS or PIXE button displays the corresponding energy spectrum and map.

No region button This removes the selected region and the entire image to be displayed when the corresponding OA-STIM, EBS and PIXE button is selected again.

Below the energy spectrum and map images are a number of rows of buttons.

Cursor channel button Moving the mouse onto the energy spectrum and left-clicking selects a channel number. Then clicking the Cursor channel button selects this position and indicates the x-cursor value to the right of the button.

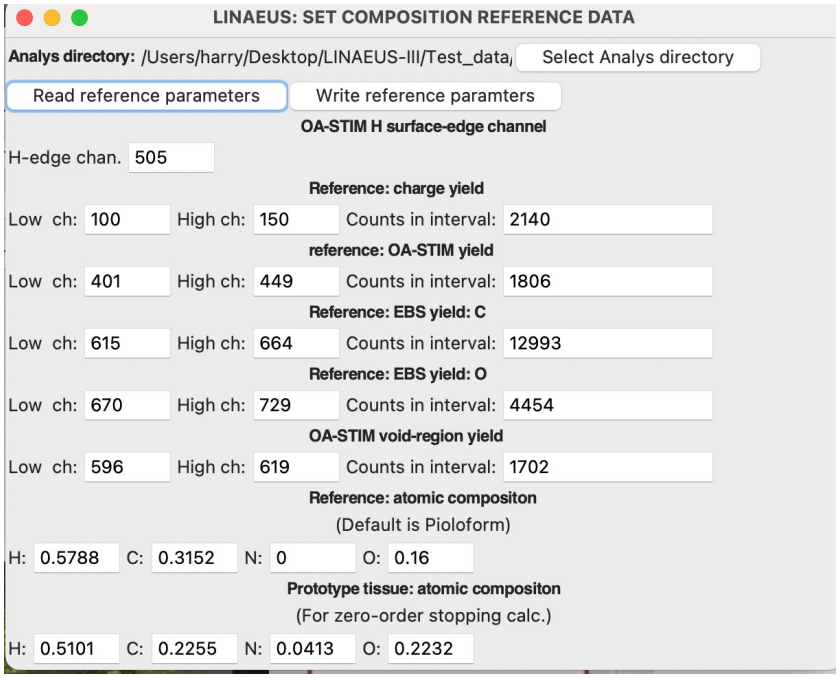

Figure 5: Screenshot of the window for setting the C, H and O reference sample composition and the composition of the prototype tissue composition.

Mark low chan. button This is used to select the lower channel of an interval. It works in the same way as the Cursor channel button.

Mark high chan. button This is used to select the lower channel of an interval. It works in the same way as the Cursor channel button. When the high and low channel numbers are selected the yield (Sum of the counts in the channel interval) is displayed along with the mean position in channels.

Use cursor chan. button The mean position is used as inputs for a number of functions. This button allows the default mean position to be overridden with the current cursor position.

Set Energy range button This sets the range of energies in channel numbers to be displayed as the interval between the high and low channels of the interval. It performs a sort and displays the resulting energy spectra and counts map for this interval.

Reset Energy range button This resets the energy range displayed to cover the entire energy range spannes (0 - 4095 channels). It performs a sort and displays the resulting energy spectra and counts map for this interval.

Save images This saves the visible energy spectrum and image in a folder "Data_visualiser_images" within the measurement folder. The OA-STIM, EBS and PIXE images are stored seperately.

Full image button This button defines a region that covers the entire image.

Draw region button This opens a new magnified image of the counts map.(Figure 9. A region can be drawn by depressing the left-mouse and moving the pointer. The freehand drawing was chosen as biological structures are rarely of regular polygons form.

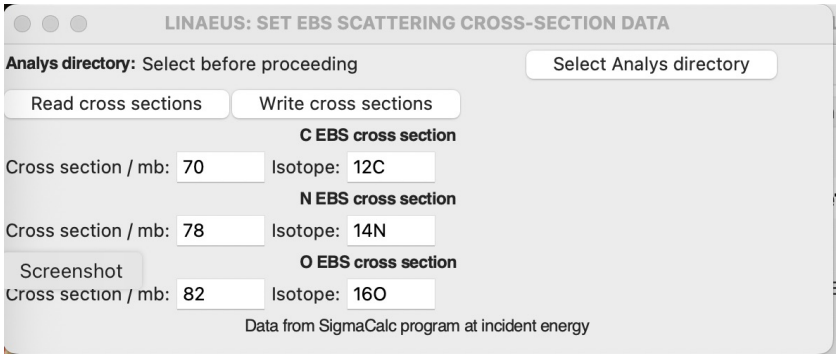

Figure 6: Screenshot of the window for setting the EBS differential cross sections for C, N and O.

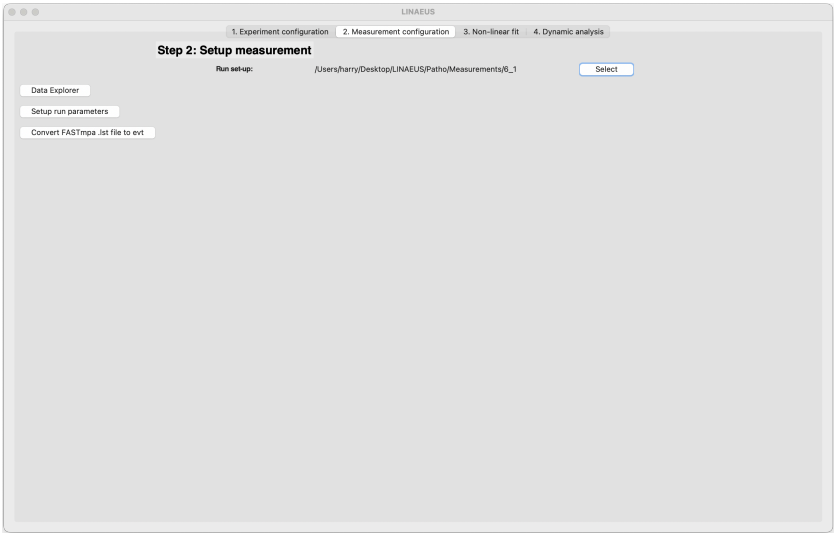

Figure 7: Screen-shot of tab for configuring the measurement parameters.

Spline-10 button This opens a new magnified image of the counts map.(Figure 9. A closed cubic spline with 10 vertices is displayed. The position of each vertices can be changed by dragging with the pointer. Depressing any keyboard key then records the shape as for Draw region.

Spline-32 button This opens a new magnified image of the counts map.(Figure 9. A closed cubic spline with 32 vertices is displayed. The position of each vertices can be changed by dragging with the pointer. Depressing any keyboard key then records the shape as for Draw region.

Region number input The number of the region is specified. (It is convenient to take region 0 as the entire image and region 1 as a void area.)

Save region as .png button The current regions is saved as a .png file with the region number in the file name. (e.g. for region 3 in a data set called "sample" the format is sample_region_3.png) This can be read in with the "Select region" button and used as a mask.

Save region as .csv button The current regions is saved as a .csv file with the region number in the file name. (e.g. for region 3 in a data set called "sample" the format is sample_region_3.png) This can be read in with the "Select region" button and used as a mask. The .csv format file can be read into a GeoPIXE region.

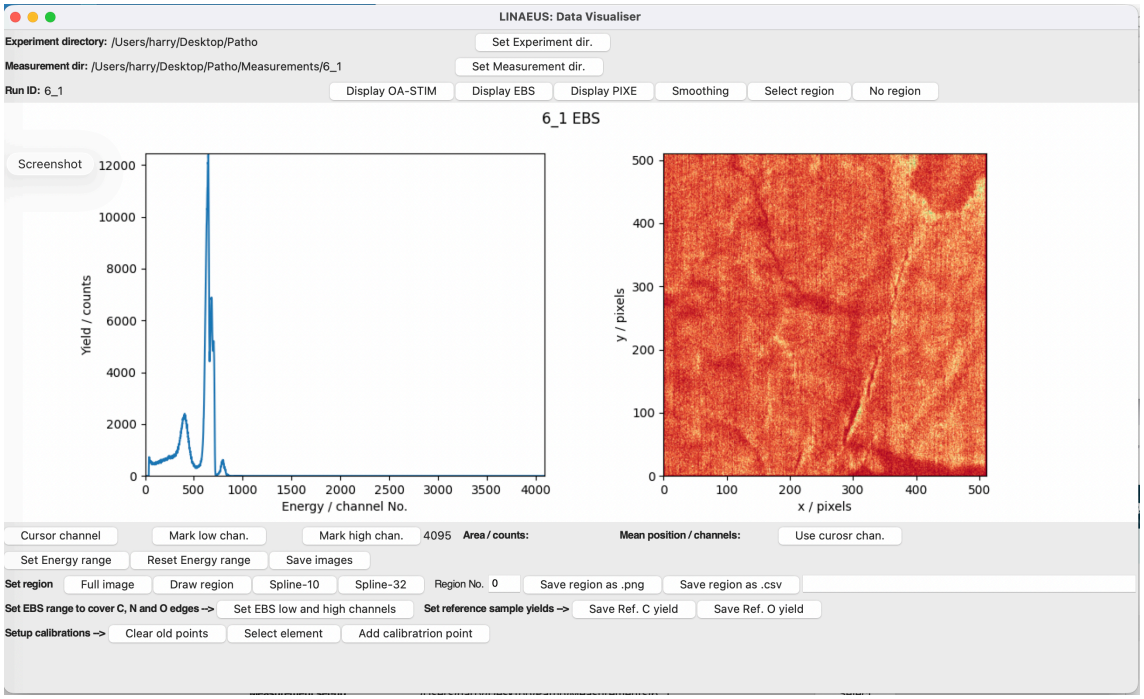

Figure 8: Screenshot of the Data Visualiser window for an EBS spectrum.

Set OA-STIM low and high channels button (OA-STIM only)
This takes the mark low chan. and Mark High cban. values as the interval over which the OA-STIM data is fitted in the Dynamic Analysis step. The energy span should cover the H peak entirely but exclude the tails from the C and O forward scattering peaks.

Get Ref H. ch. No. button (OA-STIM only)
This is used to set the channel number of the hydrogen surface. This is taken to be the mean channel number for the H-peak calculated from channel where the contents exceed 50 % of the peak height.

Get Ref H. ch. No. button (OA-STIM only)
This is intended to set the channel number of the hydrogen surface. This is taken to be the mean channel number for the H-peak calculated from channel where the contents exceed 50 % of the peak height.

Save Ref. H yield button (OA-STIM only)
This is used to save the area under the H-peak (taken to be the low chan. – high chan interval in the OA-STIM spectra to the normalisation file.

Set EBS low and high channels button (EBS only)
This takes the mark low chan. and Mark High cban. values as the interval over which the EBS data is fitted in the Dynamic Analysis step. The energy span should cover the C peak and O peaks entirely.

Save Ref. C yield button (EBS only)
This is used to save the area under the C-peak (taken to be the low chan. – high chan interval in the EBS spectra to the normalisation file.

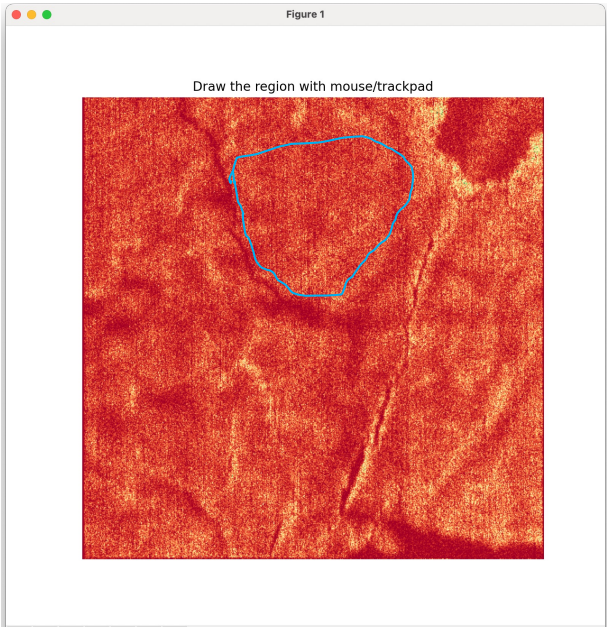

Figure 9: Screenshot of the window for draw region function.

Save Ref. O yield button (EBS only)
This is used to save the area under the O-peak (taken to be the low chan. – high chan interval in the EBS spectra to the normalisation file.

Set-up calibrations button (EBS and OA-STIM only) The "Clear old points", "Select elements" and "Add calibration point" buttons are used to set-up the energy calibrations. These need to be used in a sequence. The calibration datum can be a peak (e.g. PIXE) or an edge (EBS). The procedure to set-up the calibration is as follows. (i) Mark the edge or peak position with the Cursor. (ii) Press the Select element button to select the element on the periodicc table. (iii) Press the "Add calibration point". This does a straight line fit to the energies corresponding to scattering from the surface. (If only one point is added, the calibration line is assumed to pass through zero.) For OA-STIM and EBS the edge energy is calculated using the mass of the most abundant isotope.

Setup run parameters button This sets-up how the regions are analysed in the Dynamic Analysis step by setting parameters for each region of the sample that is measured. Figure 4 shows the window that opens. This allows the directory of files for the Measurement to be selected. The measurement ID is presented. This is used as part of the file name to identify the files generated in the Measurement directory.

Void-region data Identification of voids and subtraction of the H-background from the supporting (Pioloform) film is important to get an accurate tissue composition and thickness. The "Select void measurement file" and "Select void-region mask file" buttons allow the void region to be selected. This can be the reference Pioloform film or a region outside of the tissue region in the measurement file itself.

Adding regions for analysis The "Add new region file for analysis" section adds a file for Dynamic Analysis. Normally the "edge" position is the automatically selected to be the mean peak position. (It can also be entered manually here.) (Taking the energy difference between this and the surface peak position gives a mean thickness for the tissue.) A correction to the regional H-yield is applied from the void data in the Dynamic Analysis step to correct for the effect of H in the supporting film.

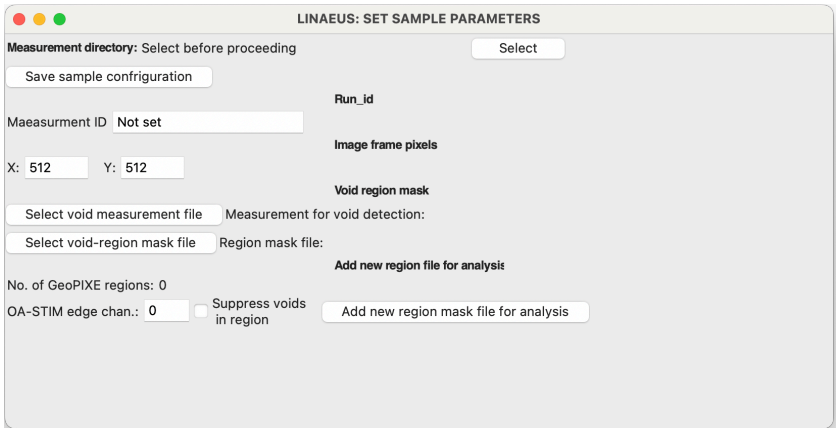

Figure 10: Screenshot of the window for entering the regional data for each measurement.

A tick in the *void-suppression box* activates identification of void region based on comparing the spatial frequency of non-zero pixels in the H-map for the data and void region.

To add the region for analysis the region file is selected. This action automatically increments the counter for the number of files to process.

Save sample configuration button This button saves the set-up data to the sample_parameters.csv file in the Measurement folder. This file is used as a control-file for batch processing of the different regions in the Dynamic Analysis step.

Convert FASTmpa .lst file to .evt button The window shown in Figure 11 is opened. This automatically finds the path to the .lst file in the Measurement directory and sets the path to the corresponding .evt form file.

Convert file button Clicking the "Convert file" button calls the C-executable program calls an external C executable program which converts the raw data format from the microbeam to an .eve event mode file. The .evt file is a human-readable file with a .csv format. Currently, provision is included to convert Fast Comtech MPA-3 format .lst format files. Extra buttons can be added to call external programs transform to .eve format data from other ion microprobe data formats such as time-stamped data and multiple dimensional sorted histograms.

The .eve file format is a human-readable file with a .csv format. It is (almost) identical to the .csv file produced by the Oxford Instruments Listmode file utility in OMDAC3 [6]. These .csv files can be read by LINEAUS

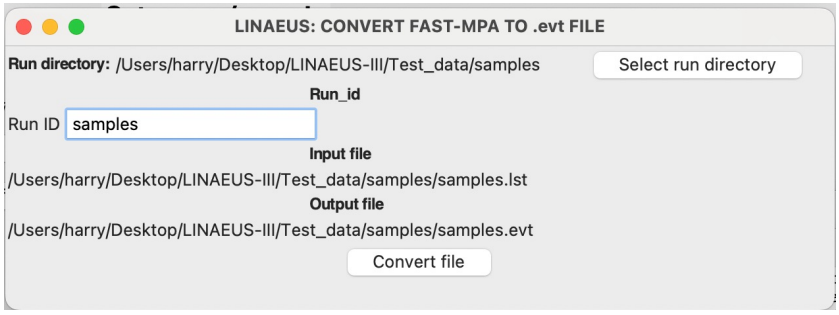

Figure 11: Screenshot of the window for producing from the .lst file from a FastCOMTEC MP3 data acquisition system to .eve format.

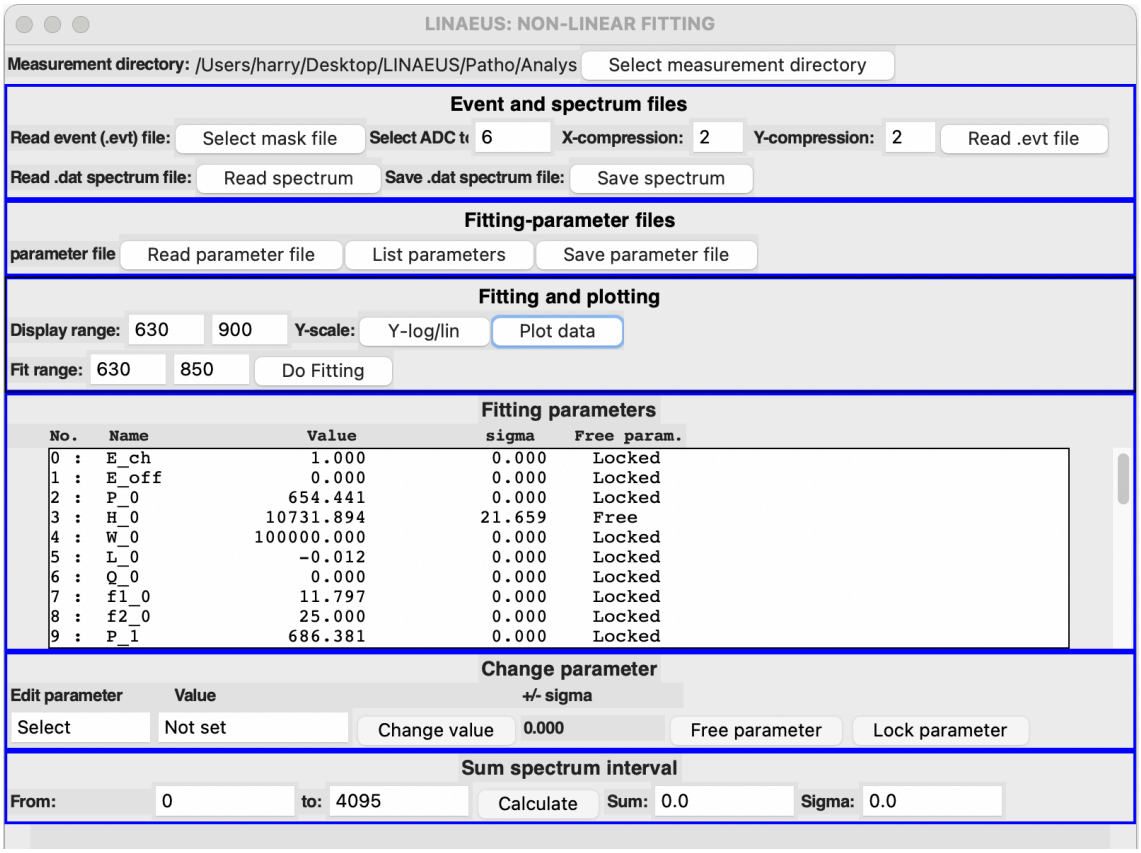

Figure 12: Screenshot of the window for the Non-linear fitting.

after changing the file extension to ".evt".) .evt formatted files are arranged as a ntuple where each row corresponds to a single detector signal. Extra buttons can be added to call external programs transform to .eve format data from other ion microprobe data formats such as time-stamped data and multi-dimensional sorted histograms.

3.4 The Non-linear fitting tab

This tab, with currently only one button, is used to call an independent Python program that perform the non-linear fitting of the prototype spectrum which is used as the basis for the Dynamic Analysis linear fitting.

Non-linear fit button The GUI window of the non-linear fitting program is shown in Figure 12. The window is divided into areas enclosed by blue rectangles that control grouped functions - for example selecting the run directory, reading the input spectrum, reading and writing parameter files, defining the fitting and plotting regions, modifying fitting parameters. The fitted function is the sum of functions that represent the energy dependence of the yield from a thick film in EBS. In addition three Gaussians can be included to represent the EBS signal from thin surface layers. Details of the prototype non-linear fitting function are given below.

The first step is to select the Measurement directory.

Event and spectrum files

Select region file button This selects the region file which is a mask applied tot the event mode data to select a region in the image. LINAEUS accepts both GeoPIXE (.csv) and LINAEUS (.png) region files.

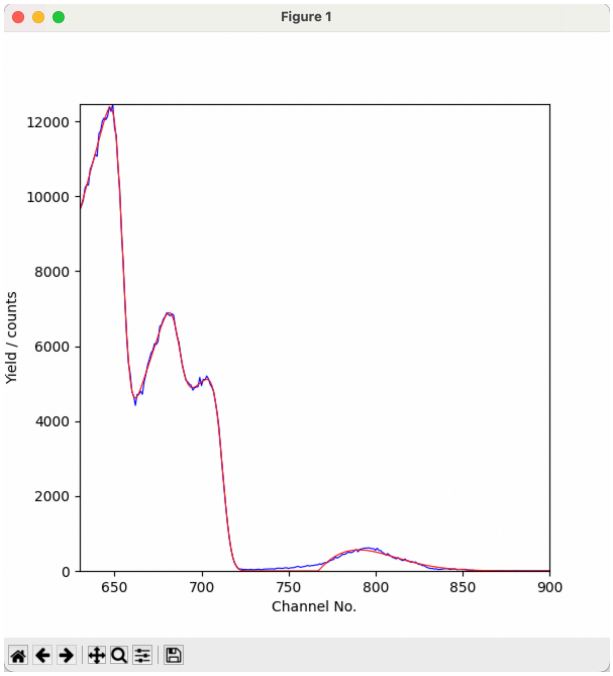

Figure 13: Screenshot of the graphical display window for example prototype EBS energy spectrum (blue) and fitted function (red).

Select ADC This is the ADC corresponding to the spectral data that is to be fitted. (For the Uppsala system 3 and 4: OA-STIM detectors, 5:PIXE and 6:EBS.)

X- and Y-compression These are the integer factors that the x- and y-ADC conversion values are divided by so that the image fits into a 512 × 512 pixel image.

Read .evt file button The .evt file name is first selected. This applies the region mask (if any) and sorts the data to produce an energy spectrum.

Reading and saving spectrum data The "Read spectrum" and "Save spectrum" buttons are used to read and save the sorted spectra. This is faster than sorting the large .evt event mode files.

Fitting parameter data The fitting parameters can be listed to the log window using the "List parameters" button. The fitting parameters can be saved and read with the "Read parameter file" and "Save parameter file" buttons.

T*he Non-linear fitting parameters to be used in the Dynamic Analysis step need to be saved to the Non_linear.par file in the Analys subdirectory.*

Fitting and plotting

Display range The range in channel numbers that are to be plotted on the display.

Y-log/lin button This toggles the Y-scale of the display between logarithmic and linear.

Plot data button This opens up a new window showing a plot of the energy spectrum. A screenshot is shown in Figure 13.

Fit range This sets the channel numbers over which the data is to be fitted. By selecting a limited range and fixing fitting parameters except the few that are sensitive to the data in the given energy span, the fit parameters can be determined precisely by the non linear fitting and then locked. This approach where different energy regions are used to fit different free-parameters effectively avoids divergence of the fit due to local minima when the number of non-linear fitting parameters is large. (viz. 16 free parameters for fitting the C, N and O EBS signals)

Do Fitting button This performs fitting of the free parameters. Locked parameters are ignored. Non-linear least-squares fitting is performed using the Minimiser function of the Python lmfit package. During the non-linear fitting procedure detailed diagnostic information from lmfit.py is written to the log window.

Fitting parameters and Change Parameter groups A scrollable list of the fitting parameters is shown in the Fitting paprameters group. This shows the name of the parameter, it's value, uncertainty and if the parameter is locked or free. When a fitting parameter in the list is selected by clicking on it, the current parameter value, uncertainty and it's locked/free status is copied to the Change parameter group.

Edit parameter field This is used to set initial values for the different fitting parameters. The naming convention is as follows:

- P_n corresponds to the high-energy position channel numbers.
- H_n the edge height in counts.
- W_n is the width in channels of the trapezium function (not used - leave at 1000.)
- L_n the slope of the trapezoid, typically -0.04.
- Q_n is a quadratic term added to the trapezoid (not-used)
- f1_n is the full width half maximum width corresponding to the high-energy edge of the trapezoid.
- f2_n is the full width half maximum width corresponding to the low-energy edge of the trapezoid (not used).

n is 0 for the C, 1 for N and 2 for O. $n = 3$ can also be used to model any tail resulting from EBS contributions for heavy elements. (E.g. See Figure 13.)

Three Gaussian functions are also included. G_P_n is the peak positions, G_A_n the areas and G_F_n the fwhmss.

Change parameter field

Edit parameter This shows the parameter name selected from the list above.

Value field The new value for the parameter is entered in this field.

Change value button This changes the value of the parameter to that in the new value and updates the list of fitting parameters.

Free parameter and lock parameter buttons These are used to free and lock the parameter selected from the list and update it.

Sum spectrum interval group (To be depreciated.) This sums the number of counts in the spectrum between the high and low channels. This function is now superseded by an corresponding function in Data Visualizer.

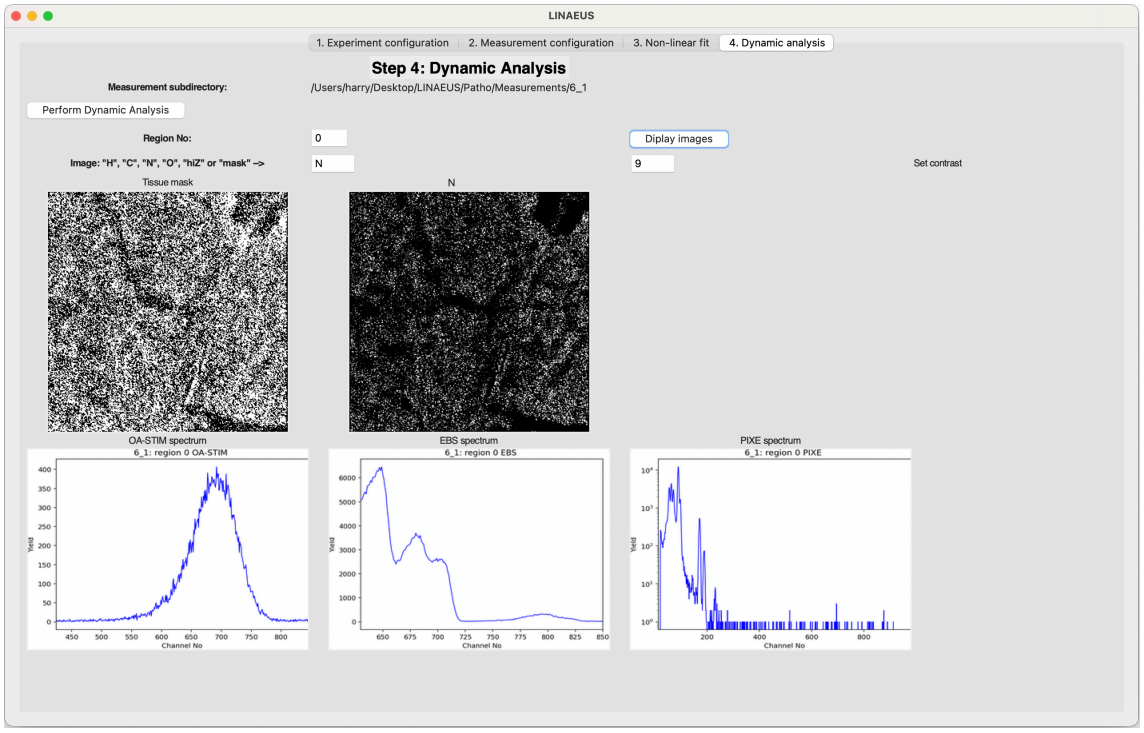

Figure 14: Screenshot of tab for performing Dynamic Analysis to determine the major element composition of different tissue-regions.

3.5 The Dynamic analysis tab

This tab performs the vast majority of the analysis tasks automatically based on the data input in the preceding tabs. If it is not specified the subdirectory for the measurement files need to be entered.

Perform Dynamic Analysis button This performs the Dynamic Analysis procedure for each of the regions specified in the "Setup run parameters" step (Measurement configuration tab). The main steps in this procedure are as follows:

1. Data from the Experiment configuration files in the Analys subdirectory are opened and read.
2. The Dynamic Analysis matrix for the C, N, O and heavier elements is calculated from the non-linear fitting parameters file data.
3. A sort is performed for the void-region .evt file data to determine the H-yield per pixel and unit charge.
4. A sort is performed on the measurement data using Direct Analysis to obtain raw elemental yield images for H, C, N and O.
5. The image is segmented into pixels corresponding to void and tissue based on the void data calculated in step 3.
6. For each region, the elemental yield images are masked to obtain the surface-edge heights for each region by sorting. If void-suppression was selected, the data is masked to include only pixels corresponding to tissue. Then the surface-edge heights for H, C, N and O are determined using the Direct Analysis matrix. (See discussion below.) Subsequently, the C, N and O surface-edge heights are transformed into an atomic composition and the thickness of the film determined from the energy difference between the H-surface energy for an infinitely thin film and the mean energy of the H-peak in the OA-STIM spectrum.

7. Finally, the elemental yield images are saved as .png files and .csv format output files of the surface-edge heights and energy difference as well as the atomic composition and thickness are written to the measurement directory (See Figure 1).

Display images button This button displays an image of the pixels that assigned as pertaining to tissue (light contrast) and the elemental yield image. In the row underneath the OA-STIM, EBS and PIXE energy spectra are displayed.

Region No., Image and Set contrast fields The Region number field selects the Region number to display. The image field selects the image to be displayed. (Valid images are H, C, N, O, High-Z and mask). The *set contrast input field* allows the contrast of the display to be changed to enhance visibility. After changing these selections the images are refreshed using the Display images button.

4 Non-linear fitting of the Prototype EBS spectrum

The protoype fitting function has been published elsewhere [13, 12, 14]. It is included here for completeness. Figure 15 illustrates how the prototype EBS elemental fitting function is made up from three element functions for C, N and O.

The yield of the element fitting function F (Figure 15 (a)) for element i is,

$$F_i(x) = \frac{1}{2} h_i \left(1 + s_i \left[p_i - x\right]\right) \mathrm{cerf}\left(2.35 \left[\frac{p_i - x}{f_i}\right]\right). \tag{5}$$

Where, x is the EBS channel number. Then, the composite prototype function (Figure 15 (ab)) is,

$$F(x) = \sum_{i=C,N,O} F_i(x). \tag{6}$$

The element function is non-linear with parameters h_i, p_i, s_i, p_i which necessitates optimisation of their values by non-linear fitting. The prototype function is a linear sum of the elemental lineshapes weighted according to the edge heights h_i.

5 The Dynamic Analysis Method

The Dynamic Analysis (DA) method follows closely that used in GeopPIXE [8][9]. The application to analysis of EBS data has been given elsewhere [13, 12, 14] but again is included here for completeness.

Consider the EBS spectrum to be represented as a 1-D matrix **S** where S_i is EBS signal counts in channel i. Here the subscript i corresponds to the channel number in the spectrum and subscript k to the number of elements/isotopes in the sample. The surface edge-heights for element n the EBS surface-edge heights a_n can be represented by a 1-D matrix **a**. The basis of the DA method is to establish the linear relation exists that relates the vectors **S** and **a**.

$$\mathbf{a} = \Gamma \mathbf{S}, \tag{7}$$

$\Gamma_{k,i}$ describes by how much the surface edge-height a_k is contributed to by each count in energy channel i. Linear least-squares regression was used to determine the matrix **a** [9]. Writing,

$$\alpha \mathbf{a} = \beta \mathbf{S}. \tag{8}$$

The β is a $k \times i$ matrix with elements;

$$\beta_{ji} = w_i \left(\frac{\partial f_i}{\partial a_j}\right). \tag{9}$$

$w_i = 1$ is the statisical weighting. (Ryan et al [9].) Then the process matrix α elements are;

$$\alpha_{jk} = \sum_i \frac{1}{w_i} \beta_{ji} \beta_{ki}. \tag{10}$$

Then the Γ matrix is given by,

$$\Gamma_{k,i} = \tilde{a}_k \sum_j \alpha^{-1}_{kj} \beta_{ji}. \tag{11}$$

$\tilde{a}_k$ is the surface-edge height from the nonlinear regression step used to determine the parameters in equations 5 and 6.

The elemental surface edge-heights for a given 2D pixel, region or an entire image can then be obtained by summing $a_{k,i}$ over the pixel, region or image. It should be noted that this $\Gamma_{k,i}$ and hence $a_{k,i}$ can be negative. (The negative values of $a_{k,i}$ arise from overlap of $F_i(E)$ so that a count in a channel can contribute positively or negatively to the corresponding edge-heights.) For spectra containing a large numbers of counts, such as a region containing a large number of pixels, this is not a problem. This is because as the number of counts increases, the edge heights will converge towards the value given by the central limit theorem. However, for small regions with few pixels and a small number of counts, statistical variations can give a negative edge heights which are unphysical. In the images produced by LINAEUS the negative pixel counts are set to zero.

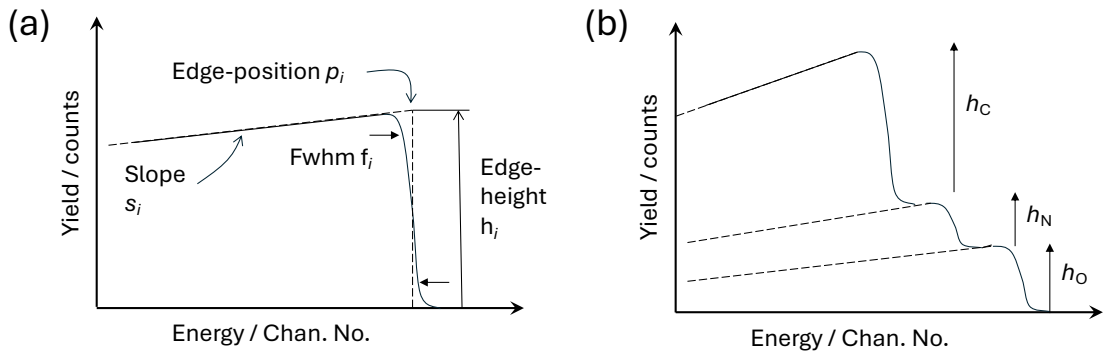

Figure 15: Schematic illustration of the prototype lineshapes for the matrix elements. (a) parameters that define the lineshape, (b) summation of lineshapes to give the composite prototype spectrum.

6 Calculation of major element composition from EBS and OA-STIM surface edge heights

The yield of counts in a peak from a thin layer of N_A atoms of element A in an ion beam analysis scattering/recoilimg measurement is given by,

$$Y_A = N_A \eta_A \frac{d\sigma_A}{d\Omega} \delta\Omega N_0. \tag{12}$$

Where, η_A is the detector efficiency, $d\sigma_A/d\Omega$ is the differential cross-section for scattering/recoiling, $\delta\Omega$ is the (small) solid angle subtended by the detector and N_0 is the number of incident ions. Consider a film of thickness t then the incident ion energy difference between the edge corresponding to $t = 0$ and a finite t is,

$$\Delta E = [\epsilon]_A N_A t. \tag{13}$$

Here, $[\epsilon]_A$ is the stopping cross-section factor which depends on A and the analysis method. For ion beam methods where the detected particle egress on the same face of the sample as the ions impinge (e.g. EBS/Rutherford Backscattering Spectrometry, glancing-angle Elastic Recoil Detection Analysis) the stopping cross-section can be written,

$$[\epsilon]_A = K_A \frac{\epsilon_{in}}{\cos\theta_1} + \frac{\epsilon_{out}}{\cos\theta_2} \tag{14}$$

θ_1 and θ_2 are the angles the incoming and outgoing ion beams make to the surface normal. OA-STIM uses a transmission geometry and then in this case the stoppign cross-section is, [12, 11],

$$[\epsilon]_A = K_A \frac{\epsilon_{out}}{\cos\theta_2} - \frac{\epsilon_{in}}{\cos\theta_1}. \tag{15}$$

The spectrum vector S_i has elements (viz. channels) that each span a constant energy difference $\mathscr{E}$, usually in units of keV. Then putting $\Delta E = \mathscr{E}$ the number of atoms per unit area of A corresponding to the energy width of one channel $\mathscr{E}$ is,

$$N_A = \frac{\mathscr{E}_A}{[\epsilon]_A}. \tag{16}$$

Then the height h_A of the spectrum will be,

$$h_A = \frac{\mathscr{E}_A}{[\epsilon]_A} \frac{d\sigma_A}{d\Omega} \delta\Omega \eta_A N_0. \tag{17}$$

Then for a compound A_mB_n the ratio of the edge-heights heights will be proportional to m/n,

$$\frac{h_A}{h_B} = \frac{m}{n} \frac{\mathscr{E}_A}{\mathscr{E}_B} \frac{[\epsilon]_B}{[\epsilon]_A} \frac{d\sigma_A}{d\sigma_B} \frac{\delta\Omega_A}{\delta\Omega_B} \frac{\eta_A}{\eta_B}. \tag{18}$$

In the case of the ratio of O and N to C measured using EBS, $\mathscr{E}_A = \mathscr{E}_B$, $\delta\Omega_A = \delta\Omega_B$ and for a Si charged particle detector $\eta_A = \eta_B$.

$$\frac{h_A}{h_B} = \frac{m}{n} \frac{[\epsilon]_B}{[\epsilon]_A} \frac{d\sigma_A}{d\sigma_B}, \tag{19}$$

which rearranging gives,

$$\frac{m}{n} = \frac{h_A}{h_B} \frac{[\epsilon]_A}{[\epsilon]_B} \frac{d\sigma_B}{d\sigma_A}, \tag{20}$$

Then considering the heights of the surface energy edges we can take the stopping cross-section on the inward and outward paths to be,

$$\epsilon_{AB_{in}} = \epsilon_{AB}(E_0) \tag{21}$$

$$\epsilon_{AB_{out}} = \epsilon_{AB}\left(K_{A,B}E_0\right) \tag{22}$$

$$\tag{23}$$

This is the surface approximation [2] Then replacing m and n by the n_C, n_N and n_O we get,

$$\frac{n_N}{n_C} = \frac{h_N}{h_C} \frac{[\epsilon]_N}{[\epsilon]_C} \frac{d\sigma_C}{d\sigma_N} \quad \text{and} \quad \frac{n_O}{n_C} = \frac{h_O}{h_C} \frac{[\epsilon]_O}{[\epsilon]_C} \frac{d\sigma_C}{d\sigma_O}. \tag{24}$$

The situation is more intricate for the hydrogen measurement from OA-STIM because the surface-edge is at lower energy than energy corresponding to H scattering/recoiling from an (infinitely) thin H-containing film This arises because in OA-STIM, the detected ions traverse the sample, leading to s smearing of the edge from the effects of energy straggling, sample thickness variations and multiple scattering. Instead, of directly measuring the H-surface edge (See e.g. Figure 5 (b) in the main text) the H-signal is linearised by assuming the H-content is constant across the thickness of the sample. Then a pseudo-surface edge-height can be assigned,

$$h_H = \frac{A_H}{\Delta E}. \tag{25}$$

Here, ΔE is the energy shift in channels of the high energy edge of H compared to an (infinitely) thin H-containing film (e.g. a thin Pioloform support film) Then the ratio of H signal height to that of C is;

$$\frac{h_H}{h_C} = \frac{n_H}{n_C} \frac{\mathscr{E}_{OA-STIM}}{\mathscr{E}_{EBS}} \frac{[\epsilon]_C}{[\epsilon]_H} \frac{d\sigma_H}{d\sigma_C} \frac{\delta\Omega_{OA-STIM}}{\delta\Omega_{EBS}} \frac{\eta_{OA-STIM}}{\eta_{EBS}}. \tag{26}$$

In this case, because two detectors are used the energy calibration, detector solid angle and detector efficiency terms do not cancel-out. Although this equation can be used directly e.g. [12], generally, it is difficult to measure detector solid angles and scattering cross sections accurately. Instead LINEAUS uses reference samples

with a known C,H, N and O contents. Then it is useful to lump the $\delta\Omega$, $d\sigma$ and η values into a calibration constant ξ so that,

$$\frac{n_H}{n_C} = \frac{h_H}{h_C}\frac{[\epsilon]_H}{[\epsilon]_C}\frac{\mathscr{E}_{OA-STIM}}{\mathscr{E}_{EBS}}\xi. \tag{27}$$

Then the atomic fractions u_j for major element j is,

$$u_j = \frac{n_j/n_C}{1 + n_H/n_C + n_N/n_C + n_O/n_C} \tag{28}$$

The value of ξ in LINAEUS is determined from the area under the peaks A_H, A_C corresponding to H and C in a reference sample such as a Pioloform film.

$$\xi_H = \frac{A_H}{A_C}\frac{C_C}{C_H}\frac{\mathscr{E}_{EBS}}{\mathscr{E}_{OA-STIM}}, \tag{29}$$

and similarly for N and O in EBS,

$$\xi_{N,O} = \frac{A_{N,O}}{A_C}\frac{C_C}{C_{N,O}}, \tag{30}$$

Since few N containing thin films are available it may be co[7]nvenient to scale ξ_N from ξ_O using the published ^{14}N(p,p)^{14}N and ^{16}O(p,p)^{16}O scattering cross-sectionsn [5, 3, 4]. This scaling to obtain ξ_N is implemented in LINAEUS.

7 PIXE signal yields

The general PIXE yield Equation [7] for a thick sample (i.e. so thick that X-ray absorption in the sample needs to tbe taken into account is,

$$Y_i = N_i N_0 \frac{\omega_v b_v t_f(E_v)\,\varepsilon(E_v)\,\Omega_{det}}{4\pi}\int_{E_0}^{E_f}\frac{\sigma_i(E)\,T_i(E)}{S_m}dE, \tag{31}$$

with,

$$T_i = \exp\left[-\frac{\mu_i}{\rho_m}\frac{1}{\cos\theta_2}\int_{E_0}^{E}\frac{dE}{S_m}\right]. \tag{32}$$

The parameters in these equations are, N_i the number of i lesser-/trace-element atoms per unit area N_0 is the number of ions, Ω_{det} the solid angle subtended by the detector. The quantities depend on the characteristic X-ray line with energy E_x are: $\sigma_i(E)$ the excitation cross section, ω_x the fluorescence yield, b_x the branching fraction, S_m the stopping force dE/dx in the matrix, $t_f(E_x)$ the transmission of the filter for X-rays with energy E_x and ε_{E_x} the detector efficiency. For Si detectors with thickness > 300μm the ε_{E_x} 100 % and is energy independent over the range 2 - 10 keV which corresponds to the K X-rays from most life elements. The transmission through the X-ray filter $t_f(E_x)$ is important because it preferentially attenuates the lower energy K X-rays in the energy range of interest. T_i is the transmission of the characteristic X-rays along the outward path. μ_i is the mass absorption coefficient of the matrix for characteristic X-rays of element i and ρ_m the density of the matrix. $S_m = dE/dx$ is the stopping of the incident ions in the matrix.

For biological tissue sections the thickness ($\sim 15\mu$m) approaches the limiting condition that the sample is so thin that (i) Energy loss in penetrating the sample does not significantly influence the ionisation cross section and (ii) That absorption of characteristic X-rays from element i in the sample is negligible. In this case the integral in the above general PIXE yield Equation becomes unity and the yield formula reduces to,

$$Y_i = N_i N_0 \frac{\omega_v \sigma_i b_v t_f(E_v)\,\varepsilon(E_v)\,\Omega_{det}}{4\pi}. \tag{33}$$

In calculating the trace element content GeoPIXE uses the full form of the PIXE yeld equation. Tissues sections of 40 μm, or so, thickness can be considered to have thickness that range from thin to intermediate from a PIXE analysis viewpoint. The two cases converge as the film thickness decreases. It is then expedient to use the thin film case for uncertainty estimates, even though the full thick target PIXE yield formula is used to calculate the trace element content.

Animal ethics statement

The subject was a cull animal from a cohort held under a holding protocol approved by the University of Louisiana at Lafayette Institutional Animal Care and Use Committee. (See Experimental section of the main text for procedure details).

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