

## Super-resolution reconstruction for two and three-dimensional LA-ICP-MS bio-imaging

Mika Westerhausen, David P. Bishop, Annette Dowd, Jonathan Wanagat, Nerida Cole, and Philip A. Doble

*Anal. Chem.*, **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.9b02380 • Publication Date (Web): 22 Oct 2019

Downloaded from [pubs.acs.org](https://pubs.acs.org) on October 24, 2019

### Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.

# Super-resolution reconstruction for two and three-dimensional LA-ICP-MS bio-imaging

Mika T. Westerhausen<sup>a</sup>, David P. Bishop<sup>a</sup>, Annette Dowd<sup>b</sup>, Jonathan Wanagat<sup>c</sup>, Nerida Cole<sup>d</sup> & Philip A. Doble<sup>a\*</sup>

- a. The Atomic Medicine Initiative, School of Mathematical and Physical Sciences, University of Technology Sydney, Broadway, NSW, 2007, Australia
- b. School of Mathematical and Physical Sciences, University of Technology Sydney, Broadway NSW, 2007, Australia
- c. Department of Medicine, Division of Geriatrics, David Geffen School of Medicine, University of California, Los Angeles, CA, USA
- d. Faculty of Science, Engineering & Technology Swinburne University of Technology, John Street, Hawthorn, Victoria, 3122, Australia

---

**ABSTRACT:** The resolution of laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) elemental bio-imaging is usually constrained by the diameter of the laser spot size and is often not adequate to explore *in situ* sub-cellular distributions of elements and / or proteins in biological tissue sections. Super-resolution reconstruction is a method typically used for many imaging modalities and combines multiple lower resolution images to create a higher resolution image. Here, we present a super-resolution reconstruction method for LA-ICP-MS imaging by ablating consecutive layers of a biological specimen with offset orthogonal scans, resulting in a 10x improvement in resolution for quantitative measurement of dystrophin in murine muscle fibres. Layer-by-layer image reconstruction was also extended to the third dimension without the requirement of image registration across multiple thin section specimens. Quantitative super-resolution reconstruction, combined with Gaussian filtering and application of the Richardson-Lucy total variation algorithm, provided superior image clarity and fidelity in two- and three-dimensions.

---

**KEYWORDS.** *Super-resolution reconstruction, Elemental bio-imaging, LA-ICP-MS, three-dimensional*

## Introduction

The resolution of an image for most practical applications of laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) using single quadrupole instruments is governed by the laser spot size, the ICP-MS total integration time, and the laser scan speed. In this context, the resolution may be defined by each sampling event of the specimen represented by each pixel of the image.

The size of a pixel in the direction of the line scan is given by  $v_l t_{sc}$  where  $v_l$  is the scan speed of the laser and  $t_{sc}$  is total integration time.<sup>1</sup> Manipulation of  $t_{sc}$  or  $v_l$  varies the resolution of the image in the direction of the line scan. For example, halving the total integration time will halve the lateral pixel size. This scenario has been frequently used to construct images with anisotropic (rectangular) pixels of improved resolution in the scan direction only, whilst the spacing between consecutive scans is defined by the diameter of the laser spot at the sample.<sup>2-5</sup> The anisotropic ratio (AR) of a pixel is given by  $AR = \frac{d_l}{v_l t_{sc}}$  (Equation 1) where  $d_l$  is the diameter of the laser spot.

Conventional LA-ICP-MS imaging has made major contributions to our understanding of the role of transition metals and other elements in health and disease at lateral resolutions of  $25 \mu\text{m}^2$  to  $2500 \mu\text{m}^2$  using laser spot sizes of 5 to  $50 \mu\text{m}$ .<sup>6,7</sup> However, there remains a pressing need to improve the resolution to adequately image subcellular localisation of bio-metals, and more recently bio-molecules via immunohistochemically assisted imaging mass spectrometry.<sup>8</sup> Here, proteins and other bio-molecular targets may be interrogated in tissues by tagging antibodies with lanthanide doped polymers<sup>9</sup> or metal nanoparticles.<sup>10</sup> A major difficulty for both of these imaging approaches is the square relationship between signal intensity and spot size, which limits the practical spot size for quadrupole-based instruments to approximately 4 to  $10 \mu\text{m}$ .<sup>5,11</sup> Below this, there is not enough material in each sampling event for detection.

Efforts to go beyond this resolution limit have involved laser oversampling and application of various deconvolution algorithms. For example, Van Malderen *et al.*<sup>12</sup> imaged the 3D distribution of <sup>55</sup>Mn in corrosion growth rings of glass at sub-micrometre resolution via overlapping spot sampling, and deconvolution with an iterative Richardson-Lucy total variation (RLTV) algorithm. An alternative approach is super resolution reconstruction (SRR) to produce high resolution images from noisy or low-resolution images. SRR is a mature technique used in many image-based fields including astronomy,<sup>13,14</sup> magnetic resonance imaging,<sup>15,16</sup> and light microscopy.<sup>17</sup> SRR reconstructs a higher resolution image by combining multiple images, which are acquired at sub-pixel distance offsets. SRR requires a non-uniform interpolation algorithm to populate pixels not filled by the lower resolution images,<sup>18,19</sup> which typically involve linear, cubic and nearest neighbor interpolation strategies.<sup>20</sup> The SRR algorithm may also be applied in three dimensions in a similar manner to MRI.<sup>21-23</sup>

3D image reconstructions by laser ablation inductively coupled mass spectrometry (LA-ICP-MS) are conventionally performed by sampling of consecutive slices

of a specimen and image registration of each of these slices for integration into a final 3D image of voxels that is a representation of the original sample.<sup>9,24-27</sup>

This work demonstrates a novel method of super resolution reconstruction (SRR) to improve the fidelity and resolution of immunohistochemically assisted quantitative LA-ICP-MS imaging with consecutive offset orthogonal raster scans in two and three dimensions. In the case of three-dimensional imaging, continuous orthogonal sampling of the specimen was undertaken until the specimen was completely ablated, eliminating image registration required by conventional approaches.

## Method

### Instrumentation

All LA-ICP-MS analyses were conducted on an Agilent 7700x series ICP-MS (Agilent Technologies, Mulgrave, Vic, Australia) coupled to a New Wave Research NWR193 (Kennelec Scientific, Mitcham, Vic, Australia) ArF excimer laser emitting at a wavelength of 193 nm. Laser ablation and ICP-MS parameters were optimised with a gelatin standard containing Gd with a laser spot size of  $15 \mu\text{m}$ , scan speed of  $30 \mu\text{m}\cdot\text{s}^{-1}$  and laser frequency of 20 Hz with the Ar carrier gas at  $1.15 \text{ L}\cdot\text{min}^{-1}$ . Calibration curves and construction of images were performed in *MassImager*, an in-house imaging data processing software package developed by Robin Schmid from the University of Münster, and FIJI used for image visualization.<sup>28</sup> The resulting calibration equations were used to convert the signal intensities of every voxel in each image to concentrations expressed as  $\text{ng}\cdot\text{g}^{-1}$ .

For solution analyses, an Agilent Technologies 7700x ICP-MS (Agilent Technologies, Mulgrave, Vic, Australia) was used with sample introduction via a micromist concentric nebuliser (Glass Expansion, West Melbourne, Vic, Australia) and a Scott type double pass spray chamber cooled to  $2^\circ\text{C}$ . ICP-MS extraction lens parameters were selected to maximise the sensitivity of a 1%  $\text{HNO}_3\text{:HCl}$  solution containing 1 ng/mL of Li, Co, Y, Ce and Tl. Helium was added into the octopole reaction cell to reduce interferences. Calibration curves were constructed and processed using Agilent Technologies Masshunter software.

### Reagents

Gadolinium (III) nitrate hexahydrate, Tris-HCl (pH 7.4), ethylenediaminetetraacetic acid (EDTA; 10 mM), polyethylene glycol ( $M_n$  400) and gelatin from bovine skin (100 mg; Type B) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia).

Grace Bio-Labs (Bend, OR, USA) supplied 6 Hybriwell™ gasket (20x9.8 mm) and clear polycarbonate cover with two ports (item number 612107, depth 0.25 mm, volume  $50 \mu\text{L}$ ). Ultrapure  $\text{HNO}_3$  and a certified Gd standard were supplied by Choice Analytical (Thornleigh, New South Wales, Australia). Anti-dystrophin monoclonal antibody (MANDYS8, sc-58754) was supplied by Santa Cruz Biosciences (Dallas, Texas, USA). MaxPar™ Labelling kit was purchased from Fluidigm (South San Francisco, CA, USA). Mouse on Mouse (M.O.M.™) Blocking Reagent (MKB-2213) was purchased from Vector Laboratories (Burlingame, CA, USA). Superblock (TBS; 37535) and TBS containing 0.1% Tween-20 (28360, TBS-T) were supplied by ThermoFisher, (Waltham, Ma, USA).

### Standard Preparation

Matrix matched gelatin standards were prepared from a modification of a previously described method.<sup>29</sup> Briefly, a stock solution of 25 mg.L<sup>-1</sup> Gd was prepared by dissolving 323.89 mg of gadolinium (III) nitrate hexahydrate in 100 mL of pH 7.4 aqueous buffer comprising 100 mM Tris-HCl, 10-mM EDTA, and 1% w/w polyethylene glycol. A series of gelatin standards were prepared by dilutions of this stock solution in the buffer to levels shown in supporting information **Error! Reference source not found.**; and addition of 100 mg of gelatin to 900µl of the dilutions at 53°C with periodic vortexing.

Flat homogeneous standard sections suitable for laser ablation were prepared by adhesion of 6 Hybriwell™ gaskets and clear polycarbonate covers with two ports to a glass slide. The slide was heated to 53°C for one minute on a dry heat block before pipetting 50 µL of the metal-gelatin standard mixture via the port.

The standard slide was cooled to room temperature for 30 minutes and then to -20°C in a freezer for 30 minutes or until the gel was frozen. The adhesive gasket and polycarbonate covers were then removed, and the standards stored at room temperature until required for use.

To determine the concentration of the standards, 100 µg of each standard was dissolved in 1 mL of HNO<sub>3</sub>, diluted to 5-mL, and analysed by solution ICP-MS. A 7-point calibration curve for quantification of the digest gelatin was constructed by diluting a 10 mg.L<sup>-1</sup> standard of Gd as per supporting information Table S2. <sup>103</sup>Rh was used as an inline internal standard.

### Sample Preparation

Wildtype C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and quadriceps muscle was dissected under guidelines of the Institutional Animal Care and Use Committee at the University of California, Los Angeles (# 2000-029-61D). All mice used in the study were male.

The MANDYS8 anti-dystrophin antibody (Santa Cruz Biotechnology) was labelled with <sup>158</sup>Gd using a MaxPar™ polymer label by the manufacturer (Fluidigm).

10 µm thick (for 2D) and 50 µm thick (3D) cryosections of mouse quadriceps were prepared for immunolabeling as follows. Air dried samples were washed in duplicate for 2 minutes with TBS at pH 7.4. Sections were then blocked with mouse on mouse blocking reagent for 60 min as per manufacturer's instructions. Sections were washed 2 x 2 mins with TBS containing 0.1% Tween-20 (TBS-T). A 1:100 dilution of the tagged anti-dystrophin antibody to give a final concentration of 2 µg.mL<sup>-1</sup> was made with Superblock® diluent. The sections were incubated with this solution for 30 minutes at room temperature. The sections were washed in triplicate for 3 minutes with TBS-T followed by an ultra-high purity water wash. Sections were air-dried and stored in a dry dust free environment until required for analysis by LA-ICP-MS.

### Optimisation of image acquisition parameters

The laser repetition frequency was maintained at 20 Hz (time between laser pulses = 50 ms) and the integrated analysis time of each plume in the ICP-MS was equal to or an integer multiple of 50 ms to minimize aliasing. Lateral

resolution improvements below the spot size of the laser was based on anisotropic oversampling by the ICP-MS in the direction of the line scan. The lateral sampling interval ( $\Delta x$ ) is a function of the total integration time i.e.,  $\Delta x = v_l t_{sc}$  (Equation 2). Seven ablation conditions were considered (Table 1) spanning various scan speeds and total integration times representing ARs from 1 to 10. The laser spot size was kept constant at 15 µm for all experiments.

Two raster pattern scans were performed for each condition in Table 1. The first data matrix was acquired from consecutive line scans from left to right for a given distance across the specimen, each line scan offset by the magnitude of the spot size of the laser (Figure 1(A)). This was followed by the collection of the second data matrix from a pattern scan in an orthogonal direction to the first, with the origin of ablation offset by half the magnitude of the spot size in the scanning directions of both layers (Figure 1 (B)). The area of ablation for each pattern was a square of 300 µm by 300 µm. The acquisition time for each raster pattern ranged from 3.4 to 13.4 mins for the two layers. Standards were ablated in the same manner before and after each acquisition scheme using the same spot size, scan speed and integration time.

**Table 1: Super resolution reconstruction acquisition parameters.**

	1	2	3	4	5	6	7
Spot Size [µm]	15	15	15	15	15	15	15
Scan Speed [µm.s <sup>-1</sup> ]	15	60	30	15	15	15	15
Total Integration Time [s]	1	0.125	0.25	0.5	0.25	0.125	0.1
Acquisition time for 300µm x 300µm area [mins]	13.4	3.4	6.7	13.4	13.4	13.4	13.4
Sampling Interval [µm]	15	7.5	7.5	7.5	3.8	1.9	1.5
Anisotropic Ratio (AR)	1	2	2	2	4	8	10

For 3D images, the sample acquisition parameters consisted of a 15 µm spot size moving at 30 µm.s<sup>-1</sup> scan speed with a total integration time of 0.125 s, equivalent to an AR of 4. All samples were 50 µm thick and were ablated in 10-layer acquisitions. Standards were ablated at the beginning, middle and end of each 10-layer batch in the same manner as the 2D samples. The optical focus was used to test the depth of field for ablation. Refocusing of layers by 5 µm of depth per layer pair was compared to unfocused ablation.

### Super Resolution Reconstruction

The two matrices described above were made conformable for addition using the Kronecker Product<sup>30</sup> of the raw data (Figure 2 A), and the row, or column matrix as applicable (Figure 2 B), resulting in matrices with equal dimensions (Figure 2 C). These matrices were then up-sampled with null values into a checkerboard pattern (Figure 2 D) and shifted by the appropriate initial offset, stacked into a 3D array and trilinearly interpolated<sup>31</sup>

(Figure 2 E). Finally, the two populated matrices were summed to create a 2D image (Figure 2 F).

Due to the depth difference between layers, step E was performed with the layers stacked on top of each other in order of ablation. For 2D images the two layers were summed together in the z-axis to create the image. Conversely, 3D images were maintained as stacks of 2D images for further processing with trilinear interpolation between the layers.

### Processing Algorithms

Two steps of post-processing were used to mitigate data convolution. The first step consisted of smoothing with a Gaussian kernel with dimensions equivalent to the spot size of the laser to correct the square pixel from circular laser beam artefacts as described previously.<sup>32</sup>

The second step consisted of minimising beam related spreading of the signal using Richardson–Lucy Total Variance Regularization (RLTV)<sup>12</sup> applied via the DeconvolutionLab 2 in Fiji.<sup>33</sup> A synthetic point spread function (PSF) comprising two Gaussians diagonally offset by the radius of the laser beam was applied, as per the original scanning pattern offset. For 3D maps, the two Gaussian PSFs were applied individually to each layer.

Quantification was performed by applying the same processing workflow to the gelatin standards in order to account for any changes to the raw data.

### Image processing software

Image processing was performed using MATLAB for SRR then FIJI for image filtering. The MATLAB code for SRR was written inhouse and is available from (<https://github.com/Elemental-Bio-Imaging-Facility>). The default FIJI Gaussian filter and the DeconvolutionLab2<sup>33</sup> plugin for Richardson-Lucy total variance deconvolution (RLTV) were used in this experiment. Processing was performed on both samples and calibration standards. 3D images were constructed using vtk files and imported into Paraview<sup>34</sup> for visualisation.

### Statistical Analysis

Statistical analyses on the raw and processed data was performed with the Real Statistics plugin for Excel.<sup>35</sup> The LODs and LOQs were estimated from the calibration curves according to the following equations:

$LOD = \frac{3\sigma}{S}$  and  $LOQ = \frac{10\sigma}{S}$ ; where  $\sigma$  was estimated by the standard error in the y-intercept and  $S$  was the slope of the calibration curve.

## Results and Discussion

### Optimisation of image acquisition parameters

We chose to use the expression of dystrophin in murine tissue as an exemplar target. Dystrophin is a protein found in muscle fibres and is part of the dystrophin-glycoprotein complex (DGC), a transmembrane multimeric complex that links the intracellular cytoskeleton and the extracellular matrix<sup>36</sup>, and provides structural stability to the sarcolemma during muscle use.<sup>37</sup> It is a low abundant protein with a clearly defined location in the muscle membrane (Figure 3), making it a suitable target for examining the improvements of SRR imaging, and the subsequent processing algorithms.

Seven scenarios were considered for investigation and optimisation of image acquisition parameters (Table 1) of various laser scan speeds ranging from 15 to 60  $\mu\text{m s}^{-1}$ , and total integration times between 0.100 and 1.00 s, representing ARs from 1 to 10, which may be thought of as a magnification factor to improve image fidelity and clarity. The spot size was maintained at 15  $\mu\text{m}$  for all experiments as preliminary scans (data not shown) indicated excellent signals of Gd for the sample under investigation, whilst spot sizes below 15  $\mu\text{m}$  did not provide sufficient intensities for subsequent SRR. Spot sizes above 15  $\mu\text{m}$  performed equally well in terms of AR magnification, however the criteria of optimisation were resolution improvement and acquisition speed.

Figure 4 depicts the resulting images from each of the seven acquisitions. Each Figure was quantified against calibration curves consisting of 5 gelatin standards (supporting information Table S1). Figures 4 (A), (B) and (C) were constructed using conventional acquisition parameters (without SRR) in which the scan speed was 1x the magnitude of the spot size, and the total integration time was 1s, equivalent to a resolution of 15  $\mu\text{m}$  per voxel and consisted of two passes without offset in the horizontal direction to maintain equivalence of ablated mass for direct comparisons of later acquisitions. Figure 4 (A) depicts the distribution of dystrophin as measured by the proxy Gd and is difficult to discern with patchy coverage and of limited utility. Figure 4 (B) shows the result of application of the Gaussian filter, and Figure 4 (C) the result of RLTV processing, respectively. As expected, each of these post acquisition processes did not improve the clarity of the image when compared against the raw image.

Consider acquisitions 2, 3 and 4, where the AR was constant at 2 (equivalent to a lateral and axial resolution of 7.5  $\mu\text{m}$  per voxel) and the scan speed was decreased from 60, 30 and 15  $\mu\text{m.s}^{-1}$ , representing increments of 4x, 2x and 1x the magnitude of the spot size. In each experiment the total integration time of the mass spectrometer was increased by a factor of the same increment in order to maintain an AR of 2.

Visual inspection of Figure 4 (D) (Acquisition 2, speed 4x) shows improved Gd coverage and emergence of the characteristic “honeycomb” structure of dystrophin within the muscle fibre membranes. Figure 4 (E) shows smoothing of the image after application of the Gaussian filter, whilst Figure 4 (F) demonstrates that the RLTV algorithm was effective for improving image clarity.

Similarly, Figure 4 (G), (H) and (I) depict the raw and post processed images (Acquisition 3, speed 2x) with half the scan speed of the former acquisition. Although the AR was maintained at 2 as before, the image clarity was superior in all three images. This improvement in clarity was a direct consequence of the washout time of the cell on this instrument (~20-50 ms) becoming decreasingly significant as a proportion of acquisition time per laser pulse. The image clarity following application of the post processing algorithms was again superior to the raw SRR image.

This effect is even more prominent in Acquisition 4 in which the total scan speed was at the same magnitude of the spot size (Figure 4 (J), (K) and (L)). Here, the distribution of dystrophin is clearly visible in the raw image and further improved by application of the Gaussian filter and the RLTV algorithm.

In general, image blur effects were acceptable at scan speeds below two times the magnitude of the spot size, i.e.  $30 \mu\text{m}\cdot\text{s}^{-1}$ . Although faster scan speeds increased the Gd signal, the image blur was generally not suitable for SRR acquisition.

Acquisition 5 demonstrates that the AR can be further increased to 4, representing a lateral and axial resolution per voxel of  $3.8 \mu\text{m}$  (Figure 4 (M), (N), and (O)). The dystrophin distribution is clearly visible in the raw image and is further clarified by application of the post processing algorithms. Acquisitions 6 (Figure 4 (P), (Q) and (R)), and 7 (Figure 4 (S), (T) and (U)) show that the AR can be further increased to 8 and 10, representing lateral and axial resolutions of 1.9 and  $1.5 \mu\text{m}$  per pixel, respectively, resulting in post processed images of excellent clarity and fidelity.

In consideration of these factors, the best compromise between resolution, acquisition time, and cell washout effects was when the scan speed was less than two times the magnitude of the spot size. This compromise is only applicable to the instrument in our laboratory as new cell designs available from various vendors have significantly reduced washout times to approximately 1 to 5 ms.<sup>38</sup>

### Calibration

The effects of the workflow and the various processing algorithms on calibration curves are shown in supporting information Figure S1. Figure S1 (A) shows a representative calibration curve, and an image panel of each of the six gelatin standards (Figure S1 (B)) obtained from ablation of a  $300 \mu\text{m}$  by  $300 \mu\text{m}$  square of the first layer in the horizontal direction with a  $15 \mu\text{m}$  spot size, scanning speed of  $30 \mu\text{m}\cdot\text{s}^{-1}$ , and a total integration time of 0.25 seconds.

In this case, the lateral resolution was  $7.5 \mu\text{m}$ , and the axial resolution was  $15 \mu\text{m}$ , representing an AR of 2. Similarly, Figure S1 (C) shows the calibration curve and the image panel of the six gelatin standards (Figure S1 (D)) at  $300 \mu\text{m} \times 300 \mu\text{m}$  ablated from the second layer diagonally offset at  $7.5 \mu\text{m}$  (half the magnitude of the spot size) from the first. A representative post-ablation raster pattern of a standard is shown in supporting information Figure S2.

The analytical figures of merit for these acquisitions and the subsequent processing algorithms are shown in supporting information Table S3.

The calibration curve of the horizontal layer had excellent linearity and a y-intercept close to the origin at 2.4 CPS, and a slope of  $0.1433 \text{ CPS}/\text{ng}\cdot\text{g}^{-1}$ . The calibration curve of the second vertical layer also had excellent linearity and a y-intercept close to the origin at 2.0 CPS, and a slope of  $0.1237 \text{ CPS}/\text{ng}\cdot\text{g}^{-1}$ . Summation of the slopes of these two curves yields a value of  $0.2670 \text{ CPS}/\text{ng}\cdot\text{g}^{-1}$ , and a y-intercept of 4.4 CPS. The LODs and LOQs were similar for both acquisitions.

The effects of the first processing step combining two offset layers of orthogonal acquisition using SRR representing an AR of 2 is shown in Figure S1 (E). The calibration curve remained linear and again was close to the origin at 12.1 CPS. The slope of the calibration curve was  $0.2659 \text{ CPS}/\text{ng}\cdot\text{g}^{-1}$  and was not significantly different (p-value 0.870) from the summation of the slopes of the two previous cases. This was consistent with an expected doubling of the slope due to summation of two layers into a single layer, i.e. two passes of the laser would be expected to double the signal intensity per voxel as twice the amount

of material was ablated when compared against a single pass.

The calibration image panel (Figure S1 (F)) now consists of square voxels made conformable by application of the Kronecker product and bilinear interpolation of null values in the product matrix. Inspection of this standard image panel clearly shows the homogeneous distribution of Gd throughout each level of standard. The LOD of  $89 \text{ ng}\cdot\text{g}^{-1}$  and the LOQ of  $270 \text{ ng}\cdot\text{g}^{-1}$  increased by approximately 15% when compared against average values from acquisitions of the single layers.

After application of the Gaussian function (Figure S1 (G)), the slope was not significantly different from the SRR (p-value 0.326), whilst the intercept, linearity, LOD and LOQ were similar to the SRR processing step, indicating that for homogeneous distributions of Gd, this smoothing function would have negligible impact on quantification. Final application of the RLTV algorithm (Figure S1 (I)), again had no significant difference in the slope compared against the Gaussian (p-value 0.117), and negligible impact on the linearity, y-intercept, LOD and LOQ. The image panel (Figure S1 (J)) appears almost identical to the Gaussian smoothed image.

The average response (CPS) and % RSDs for standards near the LOQ or above (standards 4 to 6) is shown in supporting information Table S4 for each of the described scenarios. A one-way ANOVA showed a significant difference for averages of the response in all standards when the summed responses were compared against the processed averages (p-value < 0.05). In contrast, no significant difference was observed after application of one-way ANOVA for SRR, Gaussian and RLTV (p-value 0.24 std 4; p-value 0.09 std 5; and p-value 0.07; std 6). The initial processing with SRR, which included trilinear interpolation, decreased the % RSD in all levels of standards when compared against simple summation. Subsequent application of the Gaussian function decreased the % RSD further still, whilst application of the RLTV increased the % RSD to levels like that of the SRR processing. In all three post-processing cases the % RSD were lower than the raw acquisition data. These data demonstrate that the post processing algorithms were quantitatively invariant for homogeneous standards, whilst the Gaussian filter provided the lowest % RSDs.

### Quantification

In order to determine the effect of the calibration processing algorithms on quantification of heterogeneous distributions within tissue sections, a single layer was ablated in the horizontal direction with a  $15 \mu\text{m}$  spot at  $30 \mu\text{m}\cdot\text{s}^{-1}$  and 0.25 s integration time, equivalent to an AR of 2 (acquisition 3). This same horizontal data matrix was transposed vertically (layer 2) to simulate the ablation of a second layer, offset diagonally at  $7.5 \mu\text{m}$  and subjected to each of the processing steps described above. The average concentrations and % RSD of this double layer test is shown in supporting information Table S5. This test was designed to ensure that the workflow was also quantitatively invariant for heterogeneous specimens as no significant difference would be expected for average concentrations of Gd within the post-processing algorithms, and a similar trend for % RSDs as was observed for the calibration data. For each processing step, the simulated two-layer sample

was quantified by application of the corresponding processing step to the calibration data.

As expected, application of one-way ANOVA showed there was no significant difference in average concentration of Gd between layer 1, layer 2 and the summed layers ( $p$ -value 1). However, there was a significant difference between the average concentrations when all scenarios were compared ( $p$ -value  $<0.05$ ). There was no significant difference in average concentration of Gd within post processing algorithms ( $p$ -value 0.15). As before, the % RSD was reduced after SRR processing when compared against the raw data quantification. The % RSD was further reduced after application of the Gaussian filter and increased after application of the RLTV processing step.

Supporting information Figure S3 shows the effect of each of the processing steps on image construction for the simulated scenario described above. Figure S3 (A) and (B) depict the horizontal anisotropic acquisition, and the transposed image, respectively. The image is of low clarity and characteristic "honeycomb" structure is difficult to discern. Application of SRR (Figure S3 (C)) shows the SRR composite image, demonstrating increasing clarity, whilst the Gaussian filter smoothed the image further (Figure S3 (D)). Finally, the importance of the RLTV algorithm is shown in Figure S3 (E), where the distribution of dystrophin is clearly seen.

### Three-Dimensional Reconstruction

Two scenarios were considered for three-dimensional reconstructions, single focus ablation, and refocusing the laser after each laser pass. These two scenarios were considered to examine the possibility that the collimated top hat beam shape of the laser and the depth of field for ablation may generate artefacts in areas where the sample may have different densities, or thicknesses. Two regions of  $300\ \mu\text{m} \times 300\ \mu\text{m}$  were ablated at an AR of 4 with a spot size of  $15\ \mu\text{m}$  and at  $30\ \mu\text{m}\ \text{s}^{-1}$  and a total integration time of 0.125 s, with a single focus continuous acquisition; and refocusing at each pass in increments of  $5\ \mu\text{m}$  on  $50\ \mu\text{m}$  sections. In both cases, the specimen was removed completely after 10 passes. The total acquisition time was approximately 70 minutes for each region. Each acquisition was processed with SRR, Gaussian filtering and RLTV and is shown in Figure 5. Figure 5 (A) and (B) show the planar and oblique views, whilst Figure 5 (C) shows the isometric view of the refocused laser acquisition. The distribution of dystrophin is seen throughout the 10 sections of acquisition. The continuity of the dystrophin along the muscle fibres in the third dimension was maintained, indicating that refocusing of the laser beam was a viable method for three dimensional acquisitions.

Similarly, Figure 5 (D) and (E) show the planar and oblique views, and Figure 5 (F) shows the isometric view of the single focused laser acquisition. The distribution of dystrophin is clearly seen from the planar view. The isometric view displays a generally lower signal of Gd when compared to the refocusing method possibly due to reduced ablation efficiency when not refocusing. However, the oblique view shows equivalent signal down the full 10 layers of ablated muscle fibre, and the signal difference was most likely due to sample heterogeneity. Therefore, the single focus method was comparable to the refocusing method for up to a sample thickness of  $50\ \mu\text{m}$ . The advantage of no focus is that no prior testing of ablation

depth per laser pass is required saving both sample tissue and time.

### Conclusions

In summary, these data demonstrate that the SRR, Gaussian and RLTV post processing algorithms provide superior image fidelity, with no significant differences in concentration quantification within the post-acquisition processing procedures. The average concentrations of Gd across the heterogeneous sample increased by approximately 10 %, when compared against the raw data. Although this change was significantly different at  $\alpha = 0.05$  when compared against the raw data, the processing steps would not affect the final interpretation of the distributions of dystrophin as relative quantification is required for this application. The lowest % RSD were obtained with the Gaussian filter. On the other hand, the best image clarity was obtained after RLTV, so visual interpretation of structures within tissue sections should be performed after the final RLTV processing step.

Continuous ablation of  $50\ \mu\text{m}$  thick tissue sections and methods of a single focus and refocusing of the laser at  $5\ \mu\text{m}$  increments with each pass were equivalent for three-dimensional images. When compared against conventional approaches, the single focus and refocusing methods were improved in both terms of image clarity, resolution and simplicity. The single focus method is recommended up to a sample thickness of  $50\ \mu\text{m}$  due to the simplicity of setup. The conventional approach usually requires ablation of individual sections followed by image registration and reconstruction of the final image by stacking the slices into a contiguous representation of the specimen. The SRR approach removes the requirement for serial sectioning and image registration and eliminates anomalies associated with cutting artefacts. Furthermore, the three-dimensional volume of the SRR approach is only limited by the focusing range of the laser, and could potentially be applied to whole organ imaging such as murine brain, kidney, pancreas etc.

The ablation cell wash-out was the most significant limitation for the total acquisition time of the specimen for any given experiment and was limited to laser scan speeds of approximately  $2\ d_l\ \mu\text{m}\cdot\text{s}^{-1}$ . Beyond this speed, image blur effects negated the benefits of SRR processing. It is anticipated that this image blur problem would not be significant at speeds greater than  $2\ d_l\ \mu\text{m}\cdot\text{s}^{-1}$  with new cell designs with significantly reduced washout times. Limitations of the "speed limit" of acquisitions and the maximum ARs would then migrate primarily to factors such as laser pulse frequency and speed of the mass spectrometer duty cycle, requiring the use of higher frequency laser systems and sensitive time of flight instruments.

### Ethical statement

Animal procedures were performed in accordance with the Institutional Animal Care and Use Committee at the University of California (Los Angeles, USA) (ARC #2000-029-61D).

### AUTHOR INFORMATION

#### Corresponding Author

\* Philip A. Doble

University of Technology Sydney,  
PO Box 123, Broadway  
NSW 2007, Australia  
Tel: +61 2 9514 1792;  
E-mail: philip.doble@uts.edu.au

### Author Contributions

The manuscript was written through contributions of all authors.

### Funding Sources

PAD is the recipient of Australian Research Council Discovery Grants DP170100036 and DP190102361. JW is supported by National Institutes of Health Grants R56AG060880, R01AG055518, K02AG059847, P30AG028748, and P30AR057230. DPB is supported by an Australian Research Council Discovery Early Career Researcher Award DE180100194 and the Australian-American Fulbright Commission.

### ACKNOWLEDGMENTS

The authors would like to acknowledge Robin Schmid from the University of Münster for development and continued support of *MassImager* imaging software used in this work.

### ABBREVIATIONS

LA-ICP-MS, laser ablation-inductively coupled plasma-mass spectrometry; SRR, super-resolution reconstruction; RLTV, Richardson-Lucy total variance regularization; AR, Anisotropic ratio

### SUPPORTING INFORMATION

Concentration tables of gelatin and aqueous standards. Figures of merit after application of processing methods. Average response and % RSD for calibration standards and simulated two-layer image sample. Figures of representative calibration curves and image panels, post ablation raster pattern and image processing of simulated two-layer test.

### REFERENCES

- (1) Lear, J.; Hare, D.; Adlard, P.; Finkelstein, D.; Doble, P. *J. Anal. At. Spectrom.* **2012**, *27* (1), 159.
- (2) Herrmann, A. J.; Techritz, S.; Jakubowski, N.; Haase, A.; Luch, A.; Panne, U.; Mueller, L. *Analyst* **2017**.
- (3) Drescher, D.; Giesen, C.; Traub, H.; Panne, U.; Kneipp, J.; Jakubowski, N. *Anal. Chem.* **2012**, *84* (22), 9684–9688.
- (4) Mueller, L.; Traub, H.; Jakubowski, N.; Drescher, D.; Baranov, V. I.; Kneipp, J. *Anal. Bioanal. Chem.* **2014**, *406* (27), 6963–6977.
- (5) Theiner, S.; Schreiber-Brynzak, E.; Jakupec, M. A.; Galanski, M.; Koellensperger, G.; Keppler, B. K. *Metallomics* **2016**, *8* (4), 398–402.
- (6) Hare, D. J.; New, E. J.; de Jonge, M. D.; McColl, G.

*Chem. Soc. Rev.* **2015**, *44*, 5941–5958.

- (7) New, E. J.; Wimmer, V. C.; Hare, D. J. *Cell Chem. Biol.* **2017**, No. 2017, 1–12.
- (8) González de Vega, R.; Clases, D.; Fernández-Sánchez, M. L.; Eiró, N.; González, L. O.; Vizoso, F. J.; Doble, P. A.; Sanz-Medel, A. *Anal. Bioanal. Chem.* **2019**, *411* (3), 639–646.
- (9) Paul, B.; Hare, D. J.; Bishop, D. P.; Paton, C.; Nguyen, V. T.; Cole, N.; Niedzwiecki, M. M.; Andreozzi, E.; Vais, A.; Billings, J. L.; Bray, L.; Bush, A. I.; McColl, G.; Roberts, B. R.; Adlard, P. A.; Finkelstein, D. I.; Hellstrom, J.; Hergt, J. M.; Woodhead, J. D.; Doble, P. a.; Niedzwiecki, M. M.; Andreozzi, E.; Vais, A.; Billings, J. L.; Bray, L.; Bush, A. I.; McColl, G.; Roberts, B. R.; Adlard, P. A.; Finkelstein, D. I.; Hellstrom, J.; Hergt, J. M.; Woodhead, J. D.; Doble, P. a.; Niedzwiecki, M. M.; Andreozzi, E.; Vais, A.; Billings, J. L.; Bray, L.; Bush, A. I.; McColl, G.; Roberts, B. R.; Adlard, P. A.; Finkelstein, D. I.; Hellstrom, J.; Hergt, J. M.; Woodhead, J. D.; Doble, P. a. *Chem. Sci.* **2015**, *6* (10), 5383–5393.
- (10) Hare, D. J.; Lei, P.; Ayton, S.; Roberts, B. R.; Grimm, R.; George, J. L.; Bishop, D. P.; Beavis, A. D.; Donovan, S. J.; McColl, G.; Volitakis, I.; Masters, C. L.; Adlard, P. a.; Cherny, R. a.; Bush, A. I.; Finkelstein, D. I.; Doble, P. a. *Chem. Sci.* **2014**, *5* (6), 2160.
- (11) Cruz-Alonso, M.; Fernandez, B.; Álvarez, L.; González-Iglesias, H.; Traub, H.; Jakubowski, N.; Pereiro, R. *Microchim. Acta* **2018**, *185* (1), 64.
- (12) Van Malderen, S. J. M.; van Elteren, J. T.; Vanhaecke, F. *Anal. Chem.* **2015**, *87* (12), 6125–6132.
- (13) Takeda, H.; Farsiu, S.; Christou, J.; Milanfar, P. In *The Advanced Maui Optical and Space Surveillance Technologies Conference*; 2006.
- (14) Fruchter, A. S.; Hook, R. N. *Publ. Astron. Soc. Pacific* **2002**, *114* (792), 144–152.
- (15) Dzyubachyk, O.; Tao, Q.; Poot, D. H. J. J.; Lamb, H. J.; Zeppenfeld, K.; Lelieveldt, B. P. F. F.; van der Geest, R. J. J. *Magn. Reson. Imaging* **2015**, *42* (1), 160–167.
- (16) Wang, Y. H.; Qiao, J.; Li, J. B.; Fu, P.; Chu, S. C.; Roddick, J. F. *Meas. J. Int. Meas. Confed.* **2014**, *47* (1), 946–953.
- (17) Huang, B.; Wang, W.; Bates, M.; Zhuang, X. *Science (80-. )* **2008**, *319* (5864), 810–813.
- (18) Manivannan, N.; Clymer, B. D.; Bratasz, A.; Powell, K. A. *Int. J. Biomed. Imaging* **2013**, *2013*, 1–11.
- (19) Mahmoudzadeh, A. P.; Kashou, N. H. *J. Med. Imaging* **2014**, *1* (3), 034007.
- (20) Patil, V. H.; Bormane, D. S. In *Innovations and Advanced Techniques in Computer and Information Sciences and Engineering*; Springer Netherlands: Dordrecht, 2007; pp 483–489.
- (21) Woo, J.; Bai, Y.; Roy, S.; Murano, E. Z.; Stone, M.;



- Prince, J. L. Haynor, D. R., Ourselin, S., Eds.; 2012; Vol. 143, p 83140C.
- (22) Gholipour, A.; Warfield, S. K. *MICCAI Work. Image Anal. Dev. Brain (IADB'2009)*. **2009**, 45–52.
- (23) Gholipour, A.; Estroff, J. A.; Warfield, S. K. *IEEE Trans. Med. Imaging* **2010**, 29 (10), 1739–1758.
- (24) Chirinos, J. R.; Oropeza, D. D.; Gonzalez, J. J.; Hou, H.; Morey, M.; Zorba, V.; Russo, R. E. *J. Anal. At. Spectrom.* **2014**, 29 (7), 1292–1298.
- (25) Bishop, D. P.; Grossgarten, M.; Dietrich, D.; Vennemann, A.; Cole, N.; Sperling, M.; Wiemann, M.; Doble, P. A.; Karst, U. *Anal. Methods* **2018**, 10 (8), 836–840.
- (26) Van Malderen, S. J. M.; Laforce, B.; Van Acker, T.; Nys, C.; De Rijcke, M.; de Rycke, R.; De Bruyne, M.; Boone, M. N.; De Schamphelaere, K.; Borovinskaya, O.; De Samber, B.; Vincze, L.; Vanhaecke, F. *Anal. Chem.* **2017**, 89 (7), 4161–4168.
- (27) Hare, D. J.; Lee, J. K.; Beavis, A. D.; van Gramberg, A.; George, J.; Adlard, P. a; Finkelstein, D. I.; Doble, P. A.; *Anal. Chem.* **2012**, 84 (9), 3990–3997.
- (28) Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; Tinevez, J.-Y. J.-Y.; White, D. J.; Hartenstein, V.; Eliceiri, K.; Tomancak, P.; Cardona, A.; Liceiri, K.; Tomancak, P.; A., C. *Nat. Methods* **2012**, 9 (7), 676–682.
- (29) Birka, M.; Wentker, K. S.; Lusmöller, E.; Arheilger, B.; Wehe, C. A.; Sperling, M.; Stadler, R.; Karst, U. *Anal. Chem.* **2015**, 87 (6), 3321–3328.
- (30) Loan, C. F. V. *J. Comput. Appl. Math.* **2000**, 123 (1–2), 85–100.
- (31) Garcia, D. *Comput. Stat. Data Anal.* **2010**, 54 (4), 1167–1178.
- (32) Van Malderen, S. J. M.; van Elteren, J. T.; Vanhaecke, F. *J. Anal. At. Spectrom.* **2014**, 30 (1), 119–125.
- (33) Sage, D.; Donati, L.; Soulez, F.; Fortun, D.; Schmit, G.; Seitz, A.; Guiet, R.; Vonesch, C.; Unser, M. *Methods* **2017**, 115, 28–41.
- (34) Ahrens, J.; Geveci, B.; Law, C. *Energy* **2005**, 836, 717–732.
- (35) Charles Zaiontz. [www.real-statistics.com](http://www.real-statistics.com) 2019.
- (36) Barresi, R. *J. Cell Sci.* **2006**, 119 (2), 199–207.
- (37) Ervasti, J. M.; Campbell, K. P. *Cell* **1991**, 66 (6), 1121–1131.
- (38) Van Malderen, S. J. M.; Managh, A. J.; Sharp, B. L.; Vanhaecke, F. *J. Anal. At. Spectrom.* **2016**, 31 (2), 423–439.

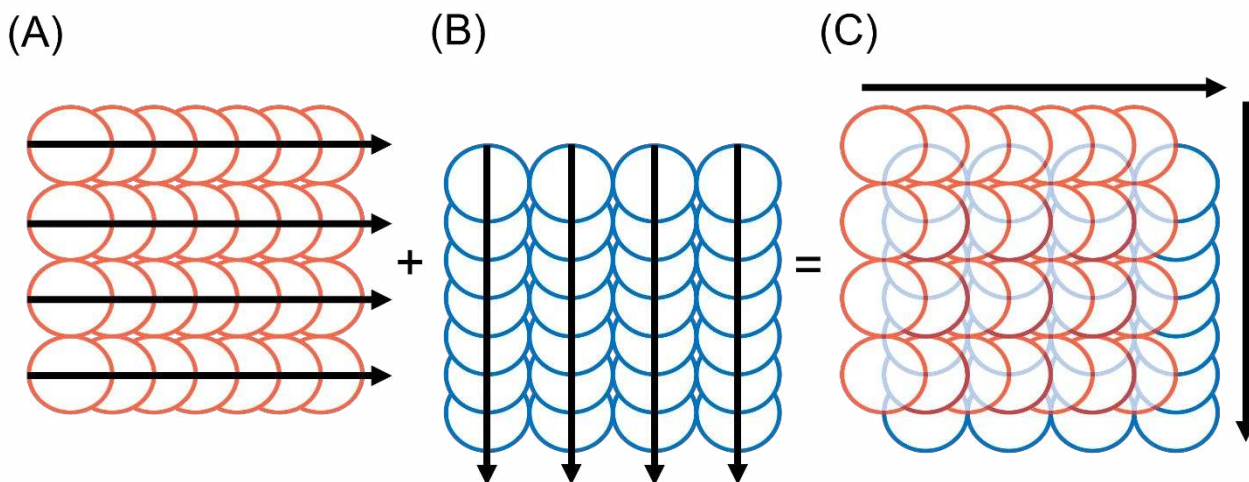


Figure 1: Orthogonal acquisition, (A) first line scans, (B) second line scans offset by half the magnitude of the laser spot size in both directions, (C) combined pattern for SRR processing. Arrows denote direction of scan.

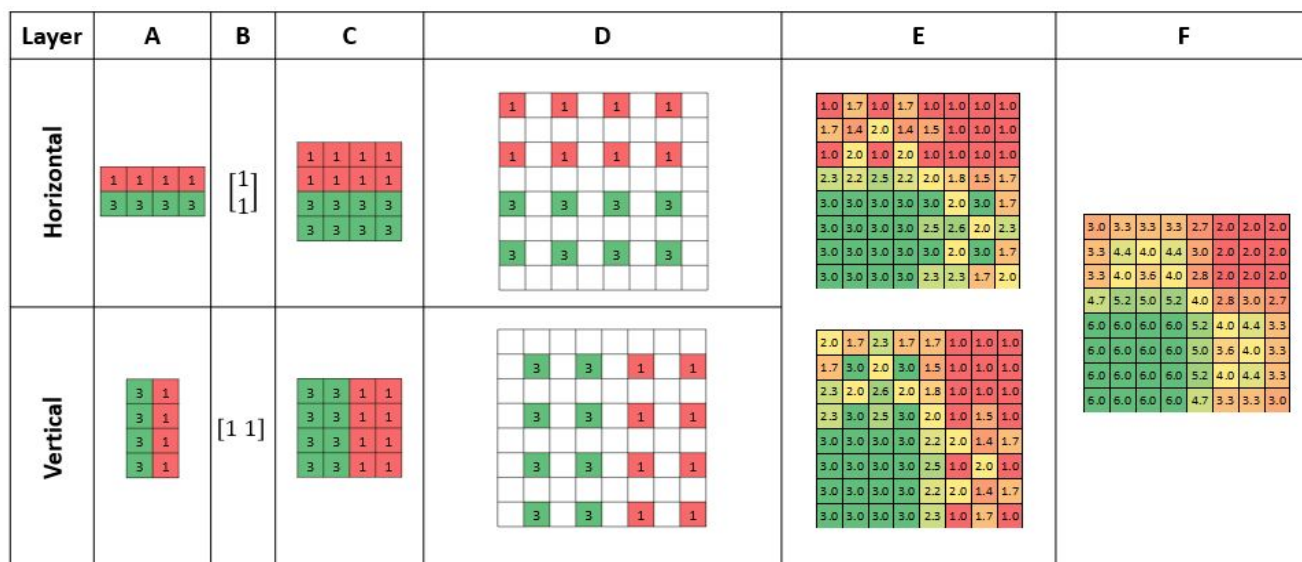


Figure 2: SRR processing of two sequentially simulated ablated layers. (A) The two layers acquired with an AR of 2 in the horizontal and vertical directions. (B) and (C) Representation of the two layers brought to congruence using the Kronecker Product. (D) Up-sampling with null values into a checkerboard pattern. (E) Layers offset and stacked into a 3D array and null values and trilinearly interpolated. (F) Populated layers summed together to produce final 2D image.

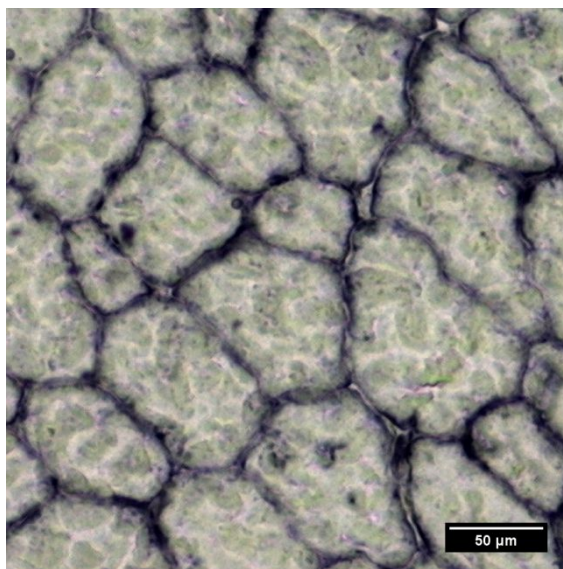
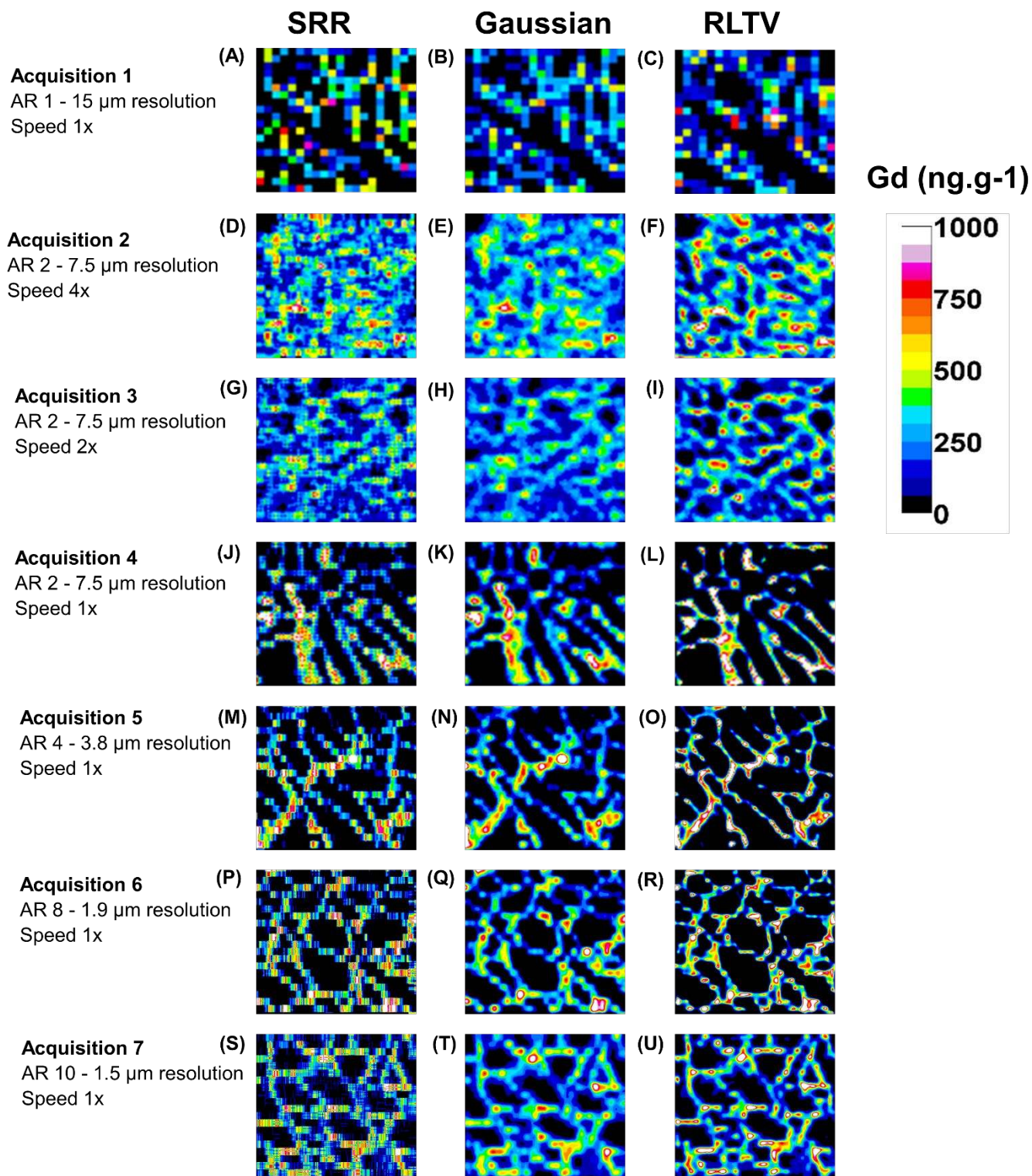


Figure 3: Representative photomicrograph of dystrophin in murine quadriceps. The expression of the protein is clearly seen as a honeycomb structure in the membranes of the muscle fibres. The protein was stained with Gd-labelled MANDYS8 primary antibody and detected using a goat anti-mouse secondary antibody (Sigma) conjugated to alkaline phosphatase with NBT/BCIP substrate (Sigma).



49 Figure 4: Image panel of acquisition parameter optimisation. Seven acquisitions were considered representing ARs ranging  
50 from 1 to 10. The SRR images are in column 1, except for (A), which was constructed in the conventional manner. Column 2  
51 contains the images after application of the Gaussian filter, whilst column 3 represents images after RLTV.



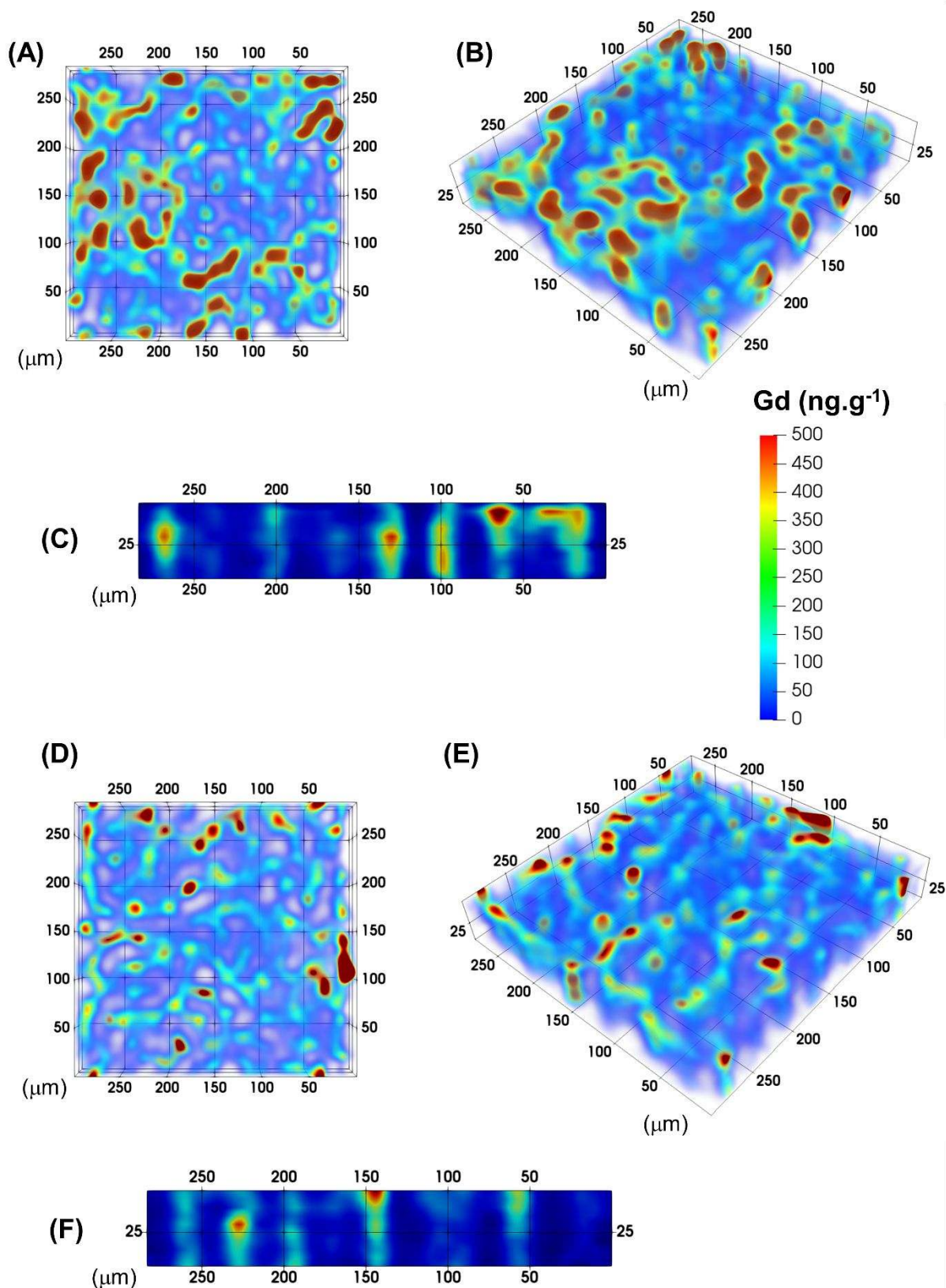
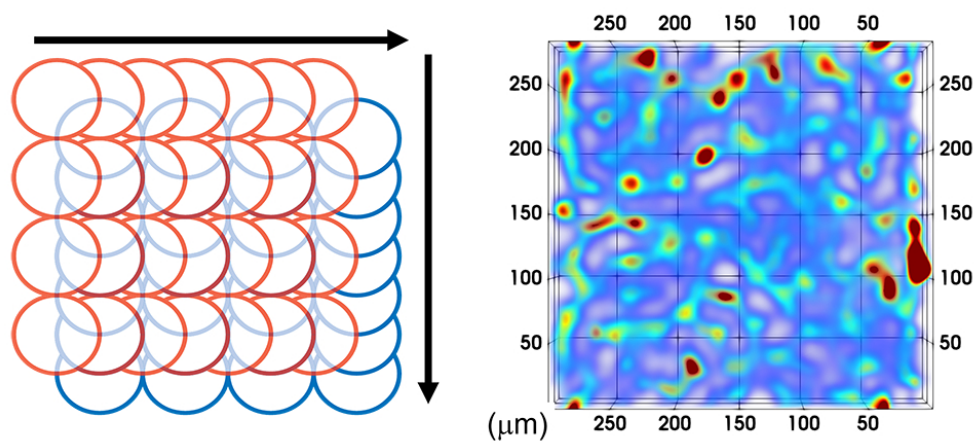


Figure 5: Three-dimensional reconstructions of continuously ablated 50  $\mu\text{m}$  section. (A) Planar view of single focus acquisition. (B) and (C) Oblique and isometric views of refocused laser acquisition at 5  $\mu\text{m}$  for each pass. (D), (E), and (F) Planar, oblique and isometric views of single focus laser acquisition. The structural integrity of the dystrophin is clearly seen throughout the 10 layers.



TOC graphic only.

82x44mm (300 x 300 DPI)