SUPPLEMENTARY INFORMATION

Criteria for edge selection in EFTEM elemental mapping

An element can have several ionization edges, some being major edges and others being minor edges. For example, cerium (Ce) has major edges at onset of 20 eV ($O_{2,3}$), 110 eV ($N_{4,5}$), 883 eV (M_5) and 901 eV (M_4); minor edges at onset of 207 eV ($N_{2,3}$), 290 eV (N_1), 1185 eV (M_3), 1273 eV (M_2) and 1435 eV (M_1)[45]. The edge onset energy can vary by about (-2 eV, +7 eV) due to bonding effects called chemical shifts, in addition to the spread due to chromatic aberration of the imaging system, which can range from few eV to tens of meV depending on the instrument[2]. The edge that is selected for elemental mapping/quantification needs to fulfill certain requirements. Firstly, the edge should be a major edge in the range ~ 100 – 2000 eV[3], the optimum EELS energy region being within 1000 eV[1]. Secondly, the edge should have a distinct shape, for e.g. a sawtooth or peaked at threshold like white-line for easy identification[3]. Finally, the edge location (in eV) should be distinct with no overlapping edge due to other adjacent elements in the periodic table.

For Ce, as described above there are potentially 4 major edges, however the first major edge $O_{2,3}$ at 20 eV does not satisfy any of the three conditions. However, using complex instrumentation like monochromators fitted to a state-of-the-art TEM and with the aid of mathematical tools such as nonlinear multivariate statistical analysis, ability to differentiate material compositions based on subtle differences in edge < 50 eV has been shown[62, 63].

The $N_{4,5}$ edge (see figure 2b and 2d) satisfies the first two conditions, it is a major peak > 100eV and has a sharp and distinct sawtooth shape. However, the $N_{4,5}$ edge for Ce is at

110 eV, and for praseodymium (Pr) it is at 113 eV, and due to the chemical shifts and the chromatic aberrations of the imaging system, unambiguous identification of the element solely based on this edge is not possible. The last two of these edges, the M_5 and the M_4 edge are very close to each other (see figure 2a and 2c), and for the purpose of elemental mapping, they are generally considered together as the $M_{4,5}$ edge. The $M_{4,5}$ edge for Ce has the so called white lines shape, that starts at ~ 883 eV and extends to ~ 920 eV. This edge is sufficiently separately from the $M_{4,5}$ edge of Pr, which starts only at ~931 eV is the preferred choice for elemental mapping/quantification. In fact, we have previously shown a multicolor EM of two astrocyte processes contacting the same synapse, with one astrocyte being labeled with Cerium conjugated DAB and the other with Praseodymium conjugated DAB[23]. It should be pointed out that, although the Cerium $M_{4,5}$ edge is explicitly separated from the Praseodymium, the extended energy-loss fine structure (EXELFS) of Ce bleeds into the Pr edge and beyond (see the area shaded in red in figure 2c), this spectral bleed-through has to be mathematically subtracted when maps of adjacent elements in the periodic table are computed[23].

Alternatively, if there is already a priori information on the elemental composition of the sample, and the primary purpose of using EELS/EFTEM is not element identification but localization or distribution of a particular element in the sample, then the criteria for the edge selection for the particular element can be slightly relaxed. For e.g., if a single cellular protein/organelle in cells was labeled by the deposition of only one lanthanide conjugated to diaminobenzidine, and the localization of this protein/organelle is to be visualized by acquiring an EFTEM elemental map of the specific lanthanide metal, in a so-called single color EM. In such a scenario, the elemental map for the lanthanide can be

potentially acquired on the intermediate-loss region of the $N_{4,5}$ edge instead of the high-loss region of the $M_{4,5}$ edge, provided that there are no overlapping edge from any of the endogenous elements in the sample.

Synthesis of Ln2-DAB

2,2'-Dibromo-6,6'-dinitrobenzidine (**2**): 2,2'-dinitrobenzidine (2.7 g, 10 mmol) was suspended in glacial acetic acid at 100°C with stirring, cooled to room temperature and bromine (1.13 ml, 22 mmol) in glacial acetic acid (10 ml) was added dropwise. After 30 mins at room temperature, LC-MS showed reaction was incomplete, so an additional 0.5 ml of bromine was added. After 30 min, water (100 ml) was added and the crude product collected by filtration, air-dried, and boiled in 95% EtOH (100 ml), cooled and re-filtered to give the product, **2** as an orange powder (2.34 g, 54%). ES-MS (m/z) $[M]^+$, $[M+ H]^+$ for $C_{12}H_9Br_2N_4O_4$, 430.9, 432.9. Found, 431.0, 433.0.

Diethyl 2,2'-dinitrobenzidine-2,2'-bis-propenoate, 3: Compound 2 (1.1 g, 2.5 mmol), ethyl acrylate (2. 5 ml, 23.5 mmol), triethylamine (1 ml, 7.1 mmol). Tri(o-tolyl) phosphine (30 mg, 0.1 mmol) and palladium acetate (10 mg, 0.045 mmol) suspended in dry DMF were heated with stirring at 80°C for 3 hours and filtered hot through a glass sinter. After evaporation to dryness, the solid was suspended in 95% EtOH (50 ml) and filtered to yield the product as a brick-red solid (1.17 g, 99%). ES-MS (m/z) $[M]^+$, $[M+ H]^+$ for $C_{22}H_{23}N_4O_8$, 471.1. Found, 471.2.

Diethyl 2,2'-dinitro-N,N'-tetra(t-butyloxycarbonyl)benzidine-2,2'-bis-propenoate, 4: Compound 3 (1.0 g, 2.1 mmol) was heated at 50°C in dry DMF (40 ml) with di-t-butyl carbonate (2.3 g, 10. 6 mmol) and DMAP (50 mg, 0.41 mmol) for 3 h. The reaction mix

was evaporated and the product purified by silica gel column chromatography by eluting with 10-25% ethyl acetate-hexane to give an oil (1.69, 92%). ES-MS (m/z) $[M]^+$, $[M+Na]^+$ for $C_{42}H_{54}N_4NaO_{16}$, 893.3. Found, 893.4.

2,2'-Diamino-N,N'-tetra(t-butyloxycarbonyl)benzidine-2,2'-bis-propanoic acid, 5: Compound 4 (1.69 g, 1.93 mmol) was hydrogenated in ethyl acetate-ethanol (1:1) with Pd/C for 6 days at rt and pressure. After filtration and evaporation to dryness, the residue was dissolved in dioxane (10 ml) and methanol (10 ml) followed by aqueous 1M- NaOH (3 ml) was then added. After overnight reaction under N_2, the organic solvents were removed by evaporation. Water and additional NaOH was then added until all solid was dissolved. After LC-MS indicated complete saponification, glacial HOAc was added dropwise to give a precipitate that was collected by filtration after chilling in ice. The product was dried over P_2O_5 in vacuo overnight to give the desired product as an off-white solid (1.40 g, 96%). ES-MS (m/z) $[M]^+$, $[M+H]^+$ for $C_{38}H_{55}N_4O_{12}$, 759.4. Found, 759.4. LC-MS showed 20% of the material has lost one BOC group during saponification. Tri-BOC, ES-MS (m/z) $[M]^+$, $[M+H]^+$ for $C_{33}H_{47}N_4O_{10}$, 659.3. Found, 659.3.

2,2'-Diamino-N,N'-tetra(t-butyloxycarbonyl)benzidine-2,2'-bis-(propanoic succinimidyl ester), 6: Compound 5 (1.40 g, ~2 mmol), N-hydroxysuccinimide (0.58 g, 5 mmol) and N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide (EDC; 0. 96 g, 5 mmol) were dissolved in dry DMF and kept overnight at room temperature. The desired products (tetra and tri-Boc) were purified by prep RP-HPLC using a water-acetonitrile-0.05% trifluoroacetic acid gradient and lyophilization of the immediately frozen fractions. Yield of off-white powder, 1.14 g (60%). Tetra-Boc, ES-MS (m/z) $[M]^+$, $[M+H]^+$ for

$C_{46}H_{61}N_6O_{16}$, 953.4. Found, 953.4. Tri-Boc, ES-MS (m/z) $[M]^+$, $[M+ H]^+$ for $C_{41}H_{53}N_6O_{14}$, 953.4. Found, 953.4.

N-(N-2-aminoethylacetamido)-ethanediamine-N,N',N'-triacetic acid, 7. EDTA free acid (161 mg, 0.55 mmol) was heated in dry DMSO (4 ml) under nitrogen with a heat gun until dissolved. After cooling to room temperature, EDC (106 mg, 0.55 mmol) was added with stirring. After 30 min, N-hydroxysuccinimide (63 mg, 0.55 mmol) was added followed by N-Boc ethylenediamine (174 ul, 1.1 mmol) after an additional 30 min. The reaction was kept overnight, evaporated to dryness and purified by prep RP-HPLC eluting with a gradient of water-acetonitrile-0.05% trifluoroacetic acid. Yield, 120 mg (27%) white solid. ES-MS (m/z) $[M]^+$, $[M+ H]^+$ for $C_{17}H_{31}N_4O_9$, 435.2. Found, 435.3. The BOC group was removed by dissolving the solid in trifluoroacetic acid for 30 min, evaporation and lyophilization of the resulting oil from 50% water-acetonitrile-0.05% trifluoroacetic acid.

Compound 8; 6 (130 mg, 0.14 mmol) was added to a solution of 7 (150 mg of BOC derivative cleaved as above, 0.35 mmol) in DMSO (1 ml) with triethylamine (0.24 ml, 1.75 mmol) at room temperature. After 24h, acetic acid (0.25 ml) was added and the product separated by prep RP-HPLC eluting with a water-acetonitrile-0.05% trifluoroacetic acid gradient. Lyophilization gave a white solid, 165 mg (84%). ES-MS (m/z) $[M]^+$, $[M+ H]^+$ for $C_{62}H_{95}N_{12}O_{24}$, 1391.7. Found, 1391.8. Boc groups were removed by treating with TFA for 15 min at rt followed by immediate evaporation. The desired product was purified by prep RP-HPLC eluting with a water-acetonitrile-0.05% trifluoroacetic acid gradient. Lyophilization gave a white solid, 80 mg. ES-MS (m/z) $[M]^+$, $[M+ H]^+$ for $C_{42}H_{63}N_{12}O_{16}$, 991.4. Found, 992.0. Titration with solutions of $CeCl_3$

using arsenazo III pH 7.2 as endpoint indicator in 100mM MOPS buffer gave 60% purity by weight indicating 6 TFA molecules are present in the resulting product.

Ln2-DAB, 9: To a 2 mg/ml (1.2 mM) solution of 8 in 0.1M sodium cacodylate pH 7.4, 25 ul $LnCl_3$ (where Ln= La, Ce, Pr, Nd; 100 mM solutions in water, except Nd was a 100mM solution in 0.1N HCl) were added per ml and the pH adjusted to pH 7.4 with 1M-NaOH.

Sample Preparation

Mitochondrial matrix-APEX2 labeled with Ce2-DAB (second generation lanthanide DAB) and DAB

HEK293T cells were cultured on imaging plates containing poly-d-lysine coated glass bottom No. 0 coverslips (P35GC-0-14C, MatTek Corporation). Cells were transiently transfected with Mitochondrial matrix-APEX2 fusion using Lipofectamine 3000 (Life Technologies). APEX2 was fused to C- terminal fusion of mito matrix[5]. After 16 hours transfection, cells were fixed with 2% glutaraldehyde (18426, Ted Pella Incorporated) in 0.1M sodium cacodylate buffer, pH 7.4 (18851, Ted Pella Incorporated) containing 1 mM $CaCl_2$ for 5 minutes at 37^{o}C and then on ice for 55 minutes. Fixative was removed and cells were rinsed with 0.1M sodium cacodylate buffer pH 7.4 (5X1min) on ice. On a set of plates an enzymatic reaction with 1.2 mM Ce2-DAB with 5 mM H_2O_2 (from 30% stock) in 0.1M sodium cacodylate buffer, pH 7.4 containing 1mM $CaCl_2$ buffer solution at pH 7.4 for 5 minutes was completed. On a separate control plate of transfected cells was an enzymatic reaction of 2.5 mM DAB with 5 mM H_2O_2 in 0.1M

sodium cacodylate buffer, pH 7.4 containing 1 mM $CaCl_2$ buffer solution at pH 7.4 for 5 minutes. After reactions, all plates of cells were rinsed with 0.1M sodium cacodylate buffer pH 7.4 (5X1min) on ice and then were posted fixed with 0.100% ruthenium tetroxide (20700-05, Electron Microscopy Sciences) containing 2 mM $CaCl_2$ and in 0.1M sodium cacodylate buffer, pH 7.4 for 20 minutes.

MiniSOG-H2B labeled with La2-DAB (second generation lanthanide DAB)

HEK293T cells were cultured on imaging plates containing poly-d-lysine coated glass bottom No. 0 coverslips (P35GC-0-14C, MatTek Corporation). Cells were transiently transfected with miniSOG-H2B fusion using Lipofectamine 3000 (Life Technologies). MiniSOG was fused to N-terminus of H2B. After 16 hours of transfection, cells were fixed with 2% glutaraldehyde (18426, Ted Pella Incorporated) in 0.1M sodium cacodylate buffer, pH 7.4 (18851, Ted Pella Incorporated) containing 1 mM $CaCl_2$ for 5 minutes at 37^{o}C and then on ice for 55 minutes. Fixative was removed, cells were rinsed with 0.1M sodium cacodylate buffer pH 7.4 (5X1min) on ice and treated for 30 min in blocking buffer consisting of 50 mM glycine, 10 mM KCN, and 5 mM aminotriazole to reduce nonspecific back-ground reaction of diaminobenzidine (DAB). Pre-equilibration with second generation La2-DAB was carried out in 0.1M sodium cacodylate buffer, pH 7.4 containing 1 mM $CaCl_2$ buffer solution at pH 7.4 that was added to the plates for 30 minutes following filtration with a 0.22 μm Millex 33mm PES sterile filter (SLGSR33RS, Sigma-Aldrich) at room temperature. Photooxidation was performed by blowing medical grade oxygen over the La2-DAB solution and cells were illuminated using a standard FITC filter set (EX470/40, DM510, BA520) with intense light from a

150W xenon lamp. Illumination was stopped as soon as a light brown precipitate occurred after 8-10 minutes. After reaction, the La2-DAB solution was removed and cells were rinsed with 0.1M sodium cacodylate buffer pH 7.4 (5X1min) on ice and then were posted fixed with 1% osmium tetroxide (19150, Electron Microscopy Sciences) containing 0.8% potassium ferrocyanide, 2mM $CaCl_2$ and in 0.1M sodium cacodylate buffer, pH 7.4 for 30 minutes.

DNA labeled with EdU and clicked with Fe-TAML-azide for oxidation of Nd-DAB2 (first generation lanthanide DAB)

Small epithelial airway cells (SEAC) were cultured on imaging plates containing poly-d-lysine coated glass bottom No. 0 coverslips (P35GC-0-14C, MatTek Corporation). SEAC were incubated with EdU (1149-100, Click Chemistry Tools) the night before for 12 hours. The cells were fixed with 2% glutaraldehyde (18426, Ted Pella Incorporated) in 0.1M sodium cacodylate buffer, pH 7.4 (18851, Ted Pella Incorporated) containing 1mM $CaCl_2$ for 5 minutes at 37^{o}C and then on ice for 55 minutes. Fixative was removed and cells were washed with 0.1M sodium cacodylate buffer pH 7.4 (5X2min) on ice, with 1X PBS (2X2min) at room temperature and rinsed 2 times quickly with 1%BSA-1XPBS by using a 0.22 μm Millex 33mm PES sterile filter (SLGSR33RS, Sigma-Aldrich) at room temperature. Cells were click reacted with 1 ml solution containing and added in the order listed, 900 μl click buffer (50 mM HEPES pH 7.6 100 mM NaCl, 0.1% saponin), 10 μl CuSO4 (100 mM), 1 μl Fe-TAML-azide (14 mM stock, giving 28 μM, Mackay et al, manuscript in preparation) and 50 μl of freshly made sodium ascorbate (100 mM) and kept covered at room temperature for 60 minutes with protection from light. During the halfway point of the click reaction, an additional 50 μl of freshly made

sodium ascorbate solution (100 mM) was added to the incubation solution. Cells were then washed with filtered 1%BSA-1XPBS (2X2min) at room temperature, 1X PBS (2X2min) at room temperature, 50 mM Bicine-100mM NaCl buffer at pH 8.3 (2X2min) and reacted with 2.5 mM Nd2-DAB in 50 mM Bicine-100mM NaCl buffer solution with 40 mM H_2O_2 (from 30%) at pH 8.3 for 15 minutes. Cells were then washed with filtered 1%BSA-1XPBS (2X1min) at room temperature, 1X PBS (2X1min) at room temperature, 50 mM Bicine-100mM NaCl buffer at pH 8.3 (2X2min), rinsed with 0.1M sodium cacodylate buffer pH 7.4 (5X1min) on ice and then were posted fixed with 1% osmium tetroxide (19150, Electron Microscopy Sciences) containing 0.8% potassium ferrocyanide, 2mM $CaCl_2$ and in 0.1M sodium cacodylate buffer, pH 7.4 for 30 minutes.

EFTEM acquisition parameters

For the high-loss EFTEM images of the mitochondrial matrix-APEX2 labeled with Ce2-DAB by the 3-window method (See figure 3e), a series of 3 images with a 100 s exposure/image, was acquired by the DE-12 detector at a frame rate of 0.04 fps and a magnification of 10kX (pixel size 0.5 nm) for each energy window. The pre-edge1, pre-edge2 and post-edge were acquired for a slit width of 30 eV at energy shifts of 815, 855 and 899 eV, respectively. The EFTEM images were acquired at a dose rate of ~ 1.2 X 10^{-4} PA/nm^2 and the total dose for the acquisition was ~ 6.8 X 10^5 e^-/nm^2.

For the intermediate-loss EFTEM images of the mitochondrial matrix-APEX2 labeled with Ce2-DAB by the 3-window method (See figure 3f), a series of 29 images with a 10 s exposure/image, was acquired by the DE-12 detector at a frame rate of 2 fps and a magnification of 10kX (pixel size 0.5 nm) for each energy window. The pre-edge1, pre-

edge2 and post-edge were acquired for a slit width of 20 eV at energy shifts of 75, 98 and 138 eV, respectively. The EFTEM images were acquired at a dose rate of ~ 1.2 X 10^{-4} PA/nm^2 and the total dose for the acquisition was ~ 6.6 X 10^5 e^-/nm^2.

For the high-loss EFTEM images of the control sample of mitochondrial matrix-APEX2 labeled with plain DAB by the 3-window method (See figure 4c), a series of 5 images with a 100 s exposure/image, was acquired by the DE-12 detector at a frame rate of 0.04 fps and a magnification of 10kX (pixel size 0.5 nm) for each energy window. The pre-edge1, pre-edge2 and post-edge were acquired for a slit width of 30 eV at energy shifts of 815, 855 and 899 eV, respectively. The EFTEM images were acquired at a dose rate of ~ 1.2 X 10^{-4} PA/nm^2 and the total dose for the acquisition was ~ 1.1 X 10^6 e^-/nm^2.

For the intermediate-loss EFTEM images of the control sample of mitochondrial matrix-APEX2 labeled with plain DAB by the 3-window method (See figure 4d), a series of 30 images with a 10 s exposure/image, was acquired by the DE-12 detector at a frame rate of 2 fps and a magnification of 10kX (pixel size 0.5 nm) for each energy window. The pre-edge1, pre-edge2 and post-edge were acquired for a slit width of 20 eV at energy shifts of 75, 98 and 138 eV, respectively. The EFTEM images were acquired at a dose rate of ~ 1.2 X 10^{-4} PA/nm^2 and the total dose for the acquisition was ~ 6.8 X 10^5 e^-/nm^2.

For the high-loss EFTEM images of the control sample of mitochondrial matrix-APEX2 labeled with plain DAB by the 3-window method (See figure 4g), a series of 5 images with a 100 s exposure/image, was acquired by the DE-12 detector at a frame rate of 0.04 fps and a magnification of 10kX (pixel size 0.5 nm) for each energy window. The pre-edge1, pre-edge2 and post-edge were acquired for a slit width of 30 eV at energy shifts of

815, 855 and 899 eV, respectively. The EFTEM images were acquired at a dose rate of ~ 1.2 X 10^{-4} PA/nm^2 and the total dose for the acquisition was ~ 1.1 X 10^6 e^-/nm^2.

For the intermediate-loss EFTEM images of the control sample of mitochondrial matrix-APEX2 labeled with plain DAB by the 3-window method (See figure 4h), a series of 30 images with a 10 s exposure/image, was acquired by the DE-12 detector at a frame rate of 2 fps and a magnification of 10kX (pixel size 0.5 nm) for each energy window. The pre-edge1, pre-edge2 and post-edge were acquired for a slit width of 15 eV at energy shifts of 84, 102 and 135 eV, respectively. The EFTEM images were acquired at a dose rate of ~ 1.2 X 10^{-4} PA/nm^2 and the total dose for the acquisition was ~ 6.8 X 10^5 e^-/nm^2.

For the intermediate-loss EFTEM images of the control sample of mitochondrial matrix-APEX2 labeled with DAB by the 3-window method (See figure 4h), a series of 30 images with a 10 s exposure/image, was acquired by the DE-12 detector at a frame rate of 2 fps and a magnification of 10kX (pixel size 0.5 nm) for each energy window. The pre-edge1, pre-edge2 and post-edge were acquired for a slit width of 15 eV at energy shifts of 84, 102 and 135 eV, respectively. The EFTEM images were acquired at a dose rate of ~ 1.2 X 10^{-4} PA/nm^2 and the total dose for the acquisition was ~ 6.8 X 10^5 e^-/nm^2.

For the intermediate-loss EFTEM images of the control sample of mitochondrial matrix-APEX2 labeled with DAB by the EFTEM SI method (See figure 4l), the stack was acquired by the DE-12 detector from energy-loss of 165 eV to 87 eV, with a slit width of 8 eV and energy step of 3 eV. The stack was acquired at a magnification of 12 kX (pixel size 0.4 nm), frame rate of 0.1 fps and exposure of 60 s per individual energy plane. The EFTEM images were acquired at a dose rate of ~ 1.8 X 10^{-4} PA/nm^2 and the total dose for the acquisition was ~ 1.9 X 10^6 e^-/nm^2.

For the intermediate-loss EFTEM images of the mitochondrial matrix-APEX2 labeled with Ce2-DAB by the EFTEM SI method (See figure 5c), the stack was acquired by the DE-12 detector from energy-loss of 150 eV to 90 eV, with a slit width of 8 eV and energy step of 3 eV. The stack was acquired at a magnification of 12 kX (pixel size 0.4 nm), frame rate of 0.1 fps and exposure of 60 s per individual energy plane. The EFTEM images were acquired at a dose rate of ~ 1.8 X 10^{-4} PA/nm^2 and the total dose for the acquisition was ~ 1.5 X 10^6 e^-/nm^2.

For the intermediate-loss EFTEM images of the MiniSOG-H2B labeled with La2-DAB by the EFTEM SI method (See figure 5g), the stack was acquired by the DE-12 detector from energy-loss of 78 eV to 144 eV, with a slit width of 8 eV and energy step of 3 eV. The stack was acquired at a magnification of 10 kX (pixel size 0.5 nm), frame rate of 0.1 fps and exposure of 60 s per individual energy plane. The EFTEM images were acquired at a dose rate of ~ 2.6 X 10^{-4} PA/nm^2 and the total dose for the acquisition was ~ 2.2 X 10^6 e^-/nm^2.

For the intermediate-loss EFTEM images of the EdU-DNA labeled with Nd-DAB2 by the EFTEM SI method (See figure 5k), the stack was acquired by the DE-12 detector from energy-loss of 96 eV to 165 eV, with a slit width of 8 eV and energy step of 3 eV. The stack was acquired at a magnification of 10 kX (pixel size 0.5 nm), frame rate of 0.1 fps and exposure of 60 s per individual energy plane. The EFTEM images were acquired at a dose rate of ~ 2.6 X 10^{-4} PA/nm^2 and the total dose for the acquisition was ~ 2.3 X 10^6 e^-/nm^2.