Chapter 4

Electron beam induced colorEM

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Nanodiamonds as multi-purpose labels for microscopy


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Abstract

Nanodiamonds containing fluorescent nitrogen-vacancy centers are increasingly attracting interest for use as a probe in biological microscopy. This interest stems from (i) strong resistance to photobleaching allowing prolonged fluorescence observation times; (ii) the possibility to excite fluorescence using a focused electron beam (cathodoluminescence; CL) for high-resolution localization; and (iii) the potential use for nanoscale sensing. For all these schemes, the development of versatile molecular labeling using relatively small diamonds is essential. Here, we show the direct targeting of a biological molecule with nanodiamonds as small as 70 nm using a streptavidin conjugation and standard antibody labelling approach. We also show internalization of 40 nm sized nanodiamonds. The fluorescence from the nanodiamonds survives osmium-fixation and plastic embedding making them suited for correlative light and electron microscopy. We show that CL can be observed from epon-embedded nanodiamonds, while surface-exposed nanoparticles also stand out in secondary electron (SE) signal due to the exceptionally high diamond SE yield. Finally, we demonstrate the magnetic read-out using fluorescence from diamonds prior to embedding. Thus, our results firmly establish nanodiamonds containing nitrogen-vacancy centers as unique, versatile probes for combining and correlating different types of microscopy, from fluorescence imaging and magnetometry to ultrastructural investigation using electron microscopy.

Introduction

In correlative microscopy, a comprehensive view on a specimen is acquired by combining information obtained with different modalities of microscopy. Arguably, correlative light and electron microscopy (CLEM)\(^1\) constitutes the most widespread form of correlative microscopy. In CLEM, fluorescence microscopy (FM) prior to EM acquisition is used, e.g., to visualize fluorescently labeled molecules within the nano-structural environment imaged with EM. Alternatively one can pinpoint a region of interest for high-resolution EM investigation using live-cell or \textit{in vivo} FM. However, the intrinsic resolution gap between FM and EM limits the degree to which molecules can be localized within the structural EM images. Preferably, this localization would be at the level of EM resolution. A major challenge in CLEM is thus to find approaches and labels that allow live-cell or \textit{in vivo} observation, maintain their fluorescence during EM sample preparation, and can be localized with near-EM resolution.

Direct electron-beam fluorescence excitation, or cathodoluminescence (CL), provides a solution that allows EM localization, but standard organic or biological fluorophores are instable under electron beam exposure\(^2\). In addition, most fluorescent labels do not survive the sample preparation needed for EM. Colloidal quantum dots are fluorescent, can be used in live cell experiments, and they can be precisely located in EM thanks to their electron dense core\(^3, 4\). However, CL from bio-conjugated quantum dots, which would allow distinguishing multiple quantum dot labels in color, has not yet been shown. This is probably due to bleaching of quantum dot fluorescence under electron exposure. With phosphor nanoparticles, CL from particles with <50 nm diameter has been observed\(^5-7\), but application in an EM-prepared sample has to our knowledge not yet been demonstrated. Larger phosphor particles doped with rare-earth atoms have also been explored for upconversion luminescence\(^8\), which may be attractive in combination with \textit{in vivo} imaging. CL from such particles after cellular uptake and sectioning for EM has been shown\(^9, 10\). However, so far only particles of >100 nm have been reported, which precludes their use as a molecular label, and conjugation schemes for these particles have not yet been reported.
Nanodiamonds as multipurpose labels for microscopy

In recent years diamond nanoparticles containing defect centers have attracted increasing interest\textsuperscript{11} for use as a molecular label because of their excellent photostability. These fluorescent nanodiamonds (FNDs) are also bio-compatible\textsuperscript{12, 13} and can be internalized in cells\textsuperscript{14-18}. Further interest in the FNDs stems from the fact that they can be used as local sensors of magnetic\textsuperscript{19} or electric fields\textsuperscript{20}, temperature\textsuperscript{21}, or strain\textsuperscript{22}, which could enable multi-parameter correlative microscopy. Moreover, stable CL from FNDs containing nitrogen-vacancy (NV) centers\textsuperscript{5}, as well as silicon-vacancy centers\textsuperscript{23} has been demonstrated, for the NV-FNDs even after cellular uptake and embedding and sectioning for scanning transmission EM\textsuperscript{24} or in live-cell EM studies\textsuperscript{25}. However, in these studies the FNDs are large (100–150 nm), limiting the use to cell uptake studies only.

Here, we take the step towards FNDs that are 40 nm and 70 nm in size on average. We show that fluorescence, optically detected magnetic resonance (ODMR), and CL can be recorded from these particles after internalization. Moreover, we present antibody-targeted labelling using the 70 nm FNDs, and demonstrate that these FNDs, targeted to a specific protein can be detected in tissue sections fixed and stained for EM using a standard protocol that allows ultrastructural preservation. Combined with live-cell fluorescence and optical recording of magnetic resonance spectra, our results demonstrate the unique potential of FNDs as biomolecular targets for multi-parameter correlative microscopy.

Material and methods

Nanodiamonds

Fluorescent nanodiamonds of 40 nm (FND40) and 70 nm (FND70) contain 10–15 and >300 NV centers, respectively as stated by the supplier (Adamas Nanotechnologies, NC, USA). FNDs were drop-casted on ITO-coated cover glasses (Optics Balzers, Liechtenstein) and subsequently air-dried. FNDs were analyzed with EM using secondary electron (SE) detection for size and dispersity on a FEI Verios scanning EM. The optimal excitation wavelength was assessed using a scanning confocal system (Zeiss LSM 780, Plan-Apochromat 63x/1.40 lens). Using lambda mode, emission spectra were recorded creating intensity profiles between 571 and 687 nm with 9 nm intervals upon excitation with 405, 440, 488, 514, 561 and 594 nm lasers with appropriate beam splitters. Intensities were measured by comparing grey values of the individual images at the 9 nm intervals using Matlab and plotted in arbitrary units. For the size measurements, images were analyzed using ImageJ. In total 5051 particles were analyzed for FND70, and 1141 for FND40. As the particles were markedly non-spherical\textsuperscript{26}, we defined the size as the square root of the surface area of FNDs as detected with secondary electrons. We note that this is an over-estimation as the shortest particle axis will mostly be perpendicular to the surface because of the drop-casting and drying. For the analysis of particle size versus CL intensity, the secondary electron images were used as a mask for the CL signal and the CL intensity of the FND was taken to be the mean signal in the mask areas. To find FNDs with zero CL intensity, the background signal plus one standard deviation was subtracted from the mean CL intensity of each ND, which was normalized afterwards.

FND40 uptake by J774 macrophages

J774A.1 macrophages (LGC Standards, Germany) which play an important role in the immune system, were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with high glucose, supplemented with 10% FBS, 1% Penicillin/streptomycin and 1% Glutamax (Gibco, ThermoFisher Scientific, The Netherlands). Cells were incubated with 1 ug/ml FND40 in cell culture medium for 5 hours at 37 °C and 5% CO\textsubscript{2}. After removal of culture medium with FNDs, cells were fixed with 4%
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paraformaldehyde/0.1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 (CaCO) for 30 minutes at room temperature (RT). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Before optically detected magnetic resonance (ODMR) measurements we used phalloidin-FITC (Sigma-Aldrich, The Netherlands) to label f-actin to visualize the cells. Samples were analyzed using a Zeiss LSM780 confocal microscope using 405 nm and 561 nm excitation. The amount of particles taken up by the cells was estimated using a home written script for the image analysis software FIJI (Fiji Is Just ImageJ, see supplementary information for a detailed explanation). Next the same samples were prepared for EM (see below).

**FND immunolabeling of HT29-EpCAM-GFP cells**

HT29 is a human epithelial colon carcinoma cell line, and HT29-EpCAM-GFP stable cells were engineered that overexpress the epithelial cell adhesion molecule (EpCAM) fused to GFP\(^{27}\). These were also cultured in DMEM complete medium. Cells were seeded in gamma irradiated 35 mm glass bottom collagen coated dishes (MatTek corporation, MA, USA) until clusters of at least 10 cells grew. Cells were fixed in 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M cacodylate (15 min, RT) and subsequently blocked in PBS with 5% BSA (PBSA). Then cells were incubated (1 hr RT) with an antibody against the extracellular domain of EpCAM, namely MOC31\(^{27,28}\). After washing in 1% PBSA, samples were incubated with rabbit-anti-mouse-biotin (Dako Netherlands) in 1% PBSA. Cells were washed in 0.1 M cacodylate and incubated with a premixed solution of FNDs and Streptavidin (Sigma-Aldrich, Zwijndrecht, the Netherlands) on a 1:20 weight ratio in 0.1 M cacodylate. As a positive control, cells were incubated with streptavidin-conjugated quantum dots (QD655, Life Technologies, The Netherlands). After washing, cells were imaged using a Zeiss LSM780 confocal microscope using a 488 nm and 561 nm laser. For every sample at least 3 different cell clusters were imaged at 3 different times. Confocal images were analyzed and processed (brightness and contrast) using Fiji\(^{29}\). EpCAM and FND signal overlap was calculated by manually removing GFP signal coming from membranes inside the cluster using FIJI software. Next the signals were subjected to a threshold and converted into binary values. Next the percentage of FND positive EpCAM pixels was calculated and related to the total of EpCAM positive pixels.

**Sample preparation for integrated light and electron microscopy**

We proceeded with the J774 and HT29 EpCAM-GFP cells described above. After washing with 0.1 M cacodylate buffer, cells were incubated with 1% osmiumtetroxide/1.5% potassiumferrocyanide in 0.1 M cacodylate buffer (30 min on ice), followed by washing with water. Next, the cells were dehydrated through an increasing graded ethanol series and left overnight in 1:1 ethanol and Epon (Serva) mixture at room temperature, which was replaced by pure Epon (4 times) and finally polymerized overnight at 58 °C. The cover glass of the imaging dish was removed using hydrogen fluoride. Areas containing cells were selected using a stereo microscope and sawn from the Epon block. Subsequently, 300 nm sections were cut with an ultramicrotome (Leica EM UC7) using a glass knife and put on an ITO coated cover glass. Subsequently, sections were counterstained with Hoechst.

**Integrated light and electron microscopy**

Fluorescence preservation was checked using the Zeiss LSM780. Next, the same sections were imaged under high vacuum using a SECOM integrated microscope (Delmic, The Netherlands) in a Zeiss Supra55 scanning EM. The SECOM is equipped with a four color LED, a dichroic mirror (Di01-R405/488/561/635, Semrock, NY, USA), a filter wheel and a CCD camera. Fluorescence of both Hoechst and FNDs was recorded using a 20x/0.75 vacuum compatible objective. EM of the same ROI was acquired using a back scattered electron detector at 10 kV with 60 μm aperture at 9.7 mm
working distance. Overlays were created using the SECOM software (Odemis). CL was recorded using a SECOM platform, only equipped with a vacuum compatible plan APO 40x/0.95 light objective and a photomultiplier tube (PMT), retrofitted to a Verios scanning EM (FEI, Eindhoven, The Netherlands). Simultaneously, CL, using the PMT, backscattered electrons, using a circular backscattered electron detector, and secondary electrons, using a through-lens detector, were recorded at either 3 keV and 0.8 nA at a 7 mm working distance. Images were processed and analyzed using Fiji and overlays were created using Adobe Photoshop.

**Figure 1 | FND applications in this study.** (a) Representation of FND40 uptake by macrophages. The diamond particles are phagocytosed by the macrophages and are transported in intracellular vesicles. (b) Immunolabeling approach: (1) the extracellular domain of EpCAM, with an intracellular GFP domain, is targeted by a monoclonal antibody (MOC-31); (2) biotinylated rabbit anti mouse IgG is used as a linker for labeling with (3) streptavidin-conjugated FND70 particles. (c) Schematic overview of the sample area of a diamond magnetometer for intracellular sensing. The cells, which contain diamond nanoparticles, are in a glass bottom petri dish. A microwire in close proximity is used to excite in the microwave regime. Simultaneously, fluorescence is collected through a microscope objective and a subsequent confocal microscope. (d) Schematic overview of integrated light and scanning EM and cathodoluminescence. (0) Primary incident electrons generate (1) backscattered electrons (BSE) and (2) secondary electrons (SE) which can be imaged in a SEM with the respective detectors. Also photons can be generated upon electron beam excitation called (3) cathodoluminescence (CL). Via an optical lens, these photons can be detected with for example a CCD camera or photo multiplier tube (PMT). Furthermore, with an integrated light and electron microscope (4) regular fluorescence imaging can be performed with photon excitation.
Optically detected magnetic resonance (ODMR) measurements

These measurements allow using the FND to read out their magnetic surrounding. Additionally, they offer a way to undoubtedly identify bright spots as FND defects. For magnetic resonance measurements a home built diamond magnetometer (similar to what is used in the community, see Fig. 1c for a schematic representation), which is a confocal microscope with built-in microwave electronics, has been used. As described previously macrophage cells where stained to identify cell borders. Borders of HT29 cells where identified via their intrinsic GFP signal. To separate the FNDs signal from the other fluorescent staining a 550 nm long pass filter was used. Signal above 550 nm was attributed to the FNDs. A laser power of 1 mW was used. After scanning an area with cells and identifying FND particles we focused on the FND spots and recorded an optically detected magnetic resonance. The frequency was swept around the expected resonance frequency of the NV centre at 2.87 GHz. This microwave signal was produced with a microwave synthesizer (Hittite HMC-T2100) sending to a homemade antenna (short circuit of a copper wire at the end of a coaxial cable, a few micrometer from the sample). Simultaneously, light intensity was collected using an Olympus UPLSAP40x2 NA = 1.3 objective and an Avalanche photodiode (SPCM-AQRF-15-FC) in single photon counting mode. The microwave power was 27 dBm and the acquisition time was 13 min.

Results and discussions

Properties of sub-100 nm FNDs

Recently, attention has gone to relatively large FNDs which show high fluorescent and CL signals. However, for bio-applications small FNDs, approaching the size of biomolecules, are preferred. First, we set out to characterize the FND40 and FND70 dispersions. When imaged with EM using secondary electron (SE) detection, the different FND types indeed show different sizes when spotted on ITO glass. However, both samples also show a substantial size variation, as can be seen for the FND70 in Fig. 2a. Figure 2b shows the size distributions measured over 5051 and 1141 particles for FND70 and FND40 respectively, which confirm the observed size polydispersity. Distributions are markedly non-Gaussian with average sizes of 54 ± 26 nm and 67 ± 37, respectively. Next, fluorescence characteristics were assessed. Excitation with 561 nm laser gave the highest emission intensity, within the red spectrum with a maximum around 660 nm. Furthermore, emission intensity increases with FND size, which can be explained by the higher amount of NV-centers in FND70 (>300) compared to FND40 (10–15). Besides light-excited fluorescence, also electron-excited CL is observed from the FNDs. CL intensity measurements (Fig. 2c) were performed simultaneously with the size measurements. These also displayed strong variations, with a few very bright FNDs and a majority of weak to dim FNDs. Note that fluorescence originates from both neutral (NV°) and negatively charged (NV°) vacancy centers, while CL has been reported to only originate from NV° centers. Spectral measurements (see Supplementary Fig. S1) confirmed NV°-only CL. FND size to CL intensity correlation (Fig. 2d) shows that brightest CL originates from the relatively larger FNDs, as may be expected from a larger number of NV-centers in bigger particles together with reduced surface quenching of excitations. However, it is also observed that through the entire size range strong particle-to-particle variations in CL intensity occur. Thus, there are relatively CL-bright small FNDs together with relatively CL-dim larger FNDs. Factors accounting for this may be the unknown out-of-plane diameter of the non-spherical FNDs (although we note that we did not observe correlation between CL and SE intensity), variation in the number of vacancy centers per particle and/or different interparticle ratios of NV° vs NV°. Despite the relatively low fluorescence of FND40, the signal/noise measurements as performed on spotted FNDs directed us to evaluate the benefits of smaller size for bio-applications.
Figure 2 | Properties of two different sizes sub 100 nm FNDs. (a) Secondary electron images of 40 nm and 70 nm FNDs. (b) Size distributions of FND40 (left) and FND70 (right) were defined as the square root of the surface area of the diamonds as detected with secondary electrons as shown in A. (c) CL intensity. (d) The correlation of diamond size with CL intensity for FND70. Bar: 1 μm.
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**FND40 uptake by macrophages visible with EM and CL**

Recently, FND ingested by different cell types have been shown\(^9,10\). We used macrophages for their uptake efficiency as a proof of principle set up for our different imaging approaches (Fig. 1). Indeed, FND40 are taken up by macrophages as assessed by their fluorescence from within the cells (Fig. 3a). Using our home written script for the FIJI software we estimated an average of 210 internalized particles per cell. The identity of the 40 nm diamonds was further confirmed by ODMR measurements revealing the presence of FND40 particles inside the cells (Fig. 3b). The characteristic NV spectrum (a double dip at the resonance frequency of 2.87 GHz) uniquely identifies diamond particles. Next, these macrophages were embedded for subsequent EM. The fluorescence of the FNDs was preserved after conventional epon embedding, including post fixation using 1% osmiumtetroxide (Fig. 3c). The retention of fluorescence could be explained since their NV-centres are embedded in the diamond structure and therefore not accessible for osmium quenching. Loss of fluorescence is often a hurdle in CLEM and maintenance is highly desired especially when an integrated light and electron microscopy approach is used (reviewed in de Boer et al.\(^1\)). Although engineered osmium-resistant fluorescent proteins and dedicated embedding protocols for fluorescence preservation exist\(^35-38\) this may come at the expense of the ultrastructure preservation. Here, however, ultrastructure is preserved, since we used conventional osmium post-fixation and epon embedding (Fig. 3d,e). Fluorescence from the EM samples is however still diffraction limited, precluding precise localization when overlaid with EM data. In order to achieve high resolution localization we utilized the CL properties of the FNDs which have been shown before with single nanodiamonds and for larger FND150 in cells\(^5, 24, 25\). Clearly, FND40 show up in CL on a dark background (Fig. 3d,e). Variations in CL intensity between different FND40 are observed, e.g., in Fig. 3d where two diamonds stand out and others appear dimmer, likely due to variations in size and number of NV centers contained in the particles. An advantage of SEM is that low energy SEs can be detected simultaneously. As diamond has an exceptionally high SE yield at few keV electron energy, which may even be more pronounced for defected diamond\(^39\), and SEs can readily escape from the small particles, the FND40 stand out particularly well in the SE image (Fig. 3d). This confirms that the higher CL originates from the relatively larger particles. Compared to the SE image, a halo around the diamonds appears in CL, which we attribute to proximity excitation due to BSE or SE excited in the tissue. When overlaid with backscattered electron (BSE) data we find localisation of FND40 particles within the ultrastructure context of macrophages. This approach reveals different stages of phagocytosis including engulfment as the plasma membrane extrudes around the particles, which are still extracellular (Fig. 3d), and FNDs in phagosomes shown by the presence of a membrane around the particles (Fig. 3e).
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(a) DAPI, Merge, FND40 images.

(b) Graph showing 10^3 Counts/s against Frequency (GHz).

(c) Hoechst, Merge, FND40 images.

(d) CL, SE, CL/SE, SE/BSE overlay images.

(e) CL, SE, CL/BSE overlay images.
Figure 3 | FND40 uptake by macrophages assessed by fluorescence microscopy, EM, magnetic resonance and CL. (a) Pre-embedding fluorescence of FND40 internalized by macrophages as seen in the diffraction interference contrast (DIC) merged picture. Nuclei are counterstained with DAPI. (b) Magnetic resonance spectra where taken at the bright spots identified as diamonds. The graph shows the spectrum taken at the red circle. 3.3% is the contrast between the resonance line and the background for 1 run. (c) Fluorescence from FND40 in a 300 nm semi-thin epon section from macrophages (M). Nuclei are counterstained with Hoechst; blue outside the cells is autofluorescence caused by epon. (d,e) CL, SE and the CL overlaid with BSE images of FND40 particles. (d) extracellular of the plasma membrane during engulfing. The lower left corner shows an overlay of CL and SE of the same image. Some particles are clearly observed with SE and not with CL. (e) FND40 particles internalized by the macrophage as they are inside vesicles. The lower left corner shows an overlay of CL and SE of the same image. Some particles are observed with SE and not with CL, but also particles observed with CL are not visible with SE. Note that single FND40 particles within one vesicle can be resolved by CL. M: macrophage; Nuc: nucleus; Intra: intracellular; Extra: extracellular; PM: plasma membrane; Mito: mitochondria; CL: cathodoluminescence; SE: secondary electrons; BSE: Backscattered electrons. Bars: (a) 10 μm, (b) 12 μm, (c) 10 μm, (d,e) 1 μm.

FND70 allow immunolabeling and superresolution detection

Given the notion that FND40 particles are detectable with CL and EM at high resolution in uptake assays, FND70 was conjugated to streptavidin for generic immunolabeling application. As a proof of principle we immuno-targeted the extracellular domain of EpCAM-GFP expressed by HT29 cells using a pre-embedding approach without permeabilization to maintain both antigenicity and ultrastructure. Immunolabeling was successful as compared to non-EpCAM-GFP expressing control cells, but sparse compared to smaller QD655 particles (~10 nm). On the other hand, FND70 can be used in ODMR (Fig. 4b). Overlap of the EpCAM positive and FND positive pixels for the cell cluster of Fig. 4a was 22.9%. This imaging mode not only allows proofing that the optical signal comes from diamond particles but also opens up the possibility to measure chemicals or certain properties in the surrounding of the particles via ODMR. Moreover, immunolabeling was detected with CL in EM samples and overlaid with BSE data for high resolution localization (Fig. 4c). Again, FNDs also stand out in SE detection (Fig. 4c). Also very dim or even non-fluorescent nanodiamonds, e.g., due to a lack of CL-active defect centers, show up in SE. On the other hand, only surface exposed particles, would be visible in SE, as for deeper (>few nanometers) lying FNDs, the low energy SEs cannot escape the sample. CL of FNDs for superresolution imaging has mostly been explored using single particles even with detecting individual defect centers within one nanodiamonds. Here, we show for the first time CL of relatively small-sized FNDs within a biological context (Figs 1d,e and 3c), compared to what others have shown before to achieve superresolution imaging. Bio-application of CL correlation with EM has been applied before with different nanoparticles, but size is often an issue.
Figure 4 | Multimodal analysis of FND70 immunolabeling of EpCAM-GFP HT29 cells. (A) Streptavidin-conjugated FND70 labels at the outside of a HT29 cell cluster. The context of different cells within the cluster is shown by GFP. Negative controls (left column) and positive controls (right column; QD655) are shown. No FND70 labeling of non-transfected negative controls is observed, context is shown by diffraction interference contrast (DIC) as EpCAM-GFP is absent. (B) Magnetic resonance spectra where taken at the bright spots identified as diamonds. The lower part of the figure shows the spectrum taken at the circled spot. 2.5% is the contrast between the resonance line and the background for 1 run. (C) CL, SE and the CL overlaid with BSE images of FND70 labeling an HT29 cell cluster at the cell surface. Note that in the right image single FND70 particles are resolved with CL. Abbreviations as in Fig. 3. Bars: (A,B) 25 μm, (C) 1 μm.
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Conclusions

FNDs have the advantage that they are stable and bleach resistant. They remain fluorescent after osmium fixation, and can also be detected using CL in samples for analysis with EM or integrated microscopes. The CL allows better localization based on the electron beam excitation rather than on the diffraction-limited light detection. Also, FNDs can be used to generate optically detected magnetic resonance signals allowing nanoscale magnetometry. We show proof-of-principle that all these FND properties can be used with small 40 nm and 70 nm particles using uptake assays or immunolabeling. Given the progress made in the last 10 years with using other nanoparticle, e.g., quantum dots, and labels for bioapplications, our results provide a first lead to further develop FNDs for life science research. This might include smaller, differently conjugated or more homogenous particle distributions. Our proof-of-principle of using multi-modal imaging with small FNDs demonstrates (i) fluorescence in EM prepared samples, (ii) CL, (iii) SE and BSD detection, and (iv) magnetometry detection, which will open up possibilities to gain additional information on the magnetic surrounding of the particles.

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References


Supplementary information

Quantification of diamonds in cells

In order to quantify internalized particles we have developed a script to be used in the image analysis software FIJI (Fiji Is Just ImageJ, https://fiji.sc/). The analysis was divided into three phases: Cell Selection, Masking and Particle Analysis. During the first phase, the images were visually inspected and random cells were selected for the analysis. Cells which aggregates associated with the cell membrane were rejected to prevent false positive results. The images were composed of several slices (z-stacks), the cell’s region was defined in all the three dimensions. In the horizontal plane, the selection considered an area containing only the cell of interest. In the height, the first and last slices containing the cell were identified. As a result, the first phase defines a volume that holds only the cell of interest. In the Masking phase, that volume is molded in order to resemble the shape of the cell. The image is converted to binary (using the Isodata algorithm to calculate the threshold) then the cell’s perimeter is detected in every slice. To find the inner volume of the cell, the program shrinks the cell’s region in order to exclude the cell membrane from the analysis. The final step uses a special function of Fiji, which analyzes the particles found in a region. Applying this function to the masked image, it is possible to directly obtain the amount of particles (connected components) in the specified region. The performance of this process is set mainly by the parameter “threshold”. The threshold is used to separate the background light from the signal emitted by the FNDs. Every pixel with intensity less than the threshold is assumed as background and deleted from the image (set as black) while every pixel with an intensity greater or equal than the threshold is assumed as part of a particle. To find an adequate value for this parameter, the image was visually inspected and different values were probed. Finally, the election was made in favor removing more background signal, but without deleting particles clearly identified, which showed no signal inside yeast cells without FNDs. As a result, the process provides the number of particles, which are found in every analyzed cell.

![Cathodoluminescence spectrum from NV0 recorded while excited with the electron beam.](image)

**Figure S1** | Cathodoluminescence spectrum from NV0 recorded while excited with the electron beam.
Chapter 4b

Multi-color electron microscopy by element-guided identification of cells, organelles and molecules

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Abstract

Cellular complexity is unraveled at nanometer resolution using electron microscopy (EM), but interpretation of macromolecular functionality is hampered by the difficulty in interpreting grey-scale images and the unidentified molecular content. We perform large-scale EM on mammalian tissue complemented with energy-dispersive X-ray analysis (EDX) to allow EM-data analysis based on elemental composition. Endogenous elements, labels (gold and cadmium-based nanoparticles) as well as stains are analyzed at ultrastructural resolution. This provides a wide palette of colors to paint the traditional grey-scale EM images for composition-based interpretation. Our proof-of-principle application of EM-EDX reveals that endocrine and exocrine vesicles exist in single cells in Islets of Langerhans. This highlights how elemental mapping reveals unbiased biomedical relevant information. Broad application of EM-EDX will further allow experimental analysis on large-scale tissue using endogenous elements, multiple stains, and multiple markers and thus brings nanometer-scale ‘color-EM’ as a promising tool to unravel molecular (de)regulation in biomedicine.
Introduction

Precise identification and localization of molecules, organelles, cells and other biological structures is a key step to unravel how these act to regulate biology. Electron microscopy (EM) provides nanometer-resolution images of the cellular ultrastructure, which can be automatically collected to allow large field-of-view or three-dimensional imaging at high magnification. However, data analysis is hampered by visual interpretation of grey-scale images, especially for rare finding or unanticipated events in large datasets. Fluorescence microscopy aids to identify biomolecules, but lacks structural context. Correlated light microscopy and EM (CLEM) allows fluorescence-guided analysis of EM data, but fluorescence retention during EM sample preparation and overlay of images differing order-of-magnitude in resolution may be technically challenging. In search for a broadly implementable technique to define molecules, organelles and cells at high resolution within mammalian tissue, we decided to implement element-guided identification using energy dispersive X-ray analysis (EDX). In mammalian tissue, detection sensitivity of typically low percent elements in combination with high count rates from carbon and oxygen as well as radiation damage have limited broad application of EDX imaging for a long time. EDX spectroscopy and imaging on cryo-fixed tissue has been pioneered by Somlyo and coworkers, and pioneering studies have mainly focused on detection of a few selected elements in small regions at relatively low resolution (see for example). Leapman and co-workers applied and pioneered electron energy loss spectroscopy (EELS) in transmission EM to discriminate cells based on sequential analysis of three elements, and recently Tsien and coworkers presented EELS-based two-color discrimination of localized deposits of lanthanides. EDX allows direct identification of many elements in parallel, either present endogenously and/or introduced by staining or labeling, at high count rates using the latest generation of silicon drift detectors (SDD). We find that this paves the way for straight-forward high-resolution elemental mapping in mammalian tissue compatible with standard EM protocols. The resulting elemental color-maps can be overlaid with the conventional EM data to allow data-mining based on composition and structure, rather than morphology only. We apply this approach in large field-of-view EM (“nanotomy”) on pancreas from a rat model for Type 1 diabetes (T1D). EDX not only allows us to identify organelles and biomolecular labels at high resolution, but also to show that distinct granules have typical elemental fingerprints. EDX-guided elemental fingerprinting in combination with large-scale EM reveals cells that contain both hormones and exocrine granules in the pancreas. Given that a sensitive EDX SDD is a standard retrofit add-on to electron microscopes, we foresee broad application of such a technique in both label free and studies using exogenous tracers. This approach is applicable to both life science and biomedical research. Such a technique increases the depth of information with the color coding of structures based on their elemental profile and brings an objective analysis tool to EM imaging.
Chapter 4b

Figure 1 | EDX defines cell-types and subcellular structures and organelles in EM images. (a) 100 nm thin cross-section of an entire rat islet of Langerhans imaged at 2.5 nm pixel size (STEM). Full high-resolution data is available via nanotomy.org (see suppl. material). (b) Area of interest (indicated in a; cyan dot) shows parts of four cells with different granules based on grey levels and morphology. (c–f) Elemental content in the ROI as indicated. (g) Overlay of N (red), P (green) and S (blue) allows identification of cells and granules based on elemental content. (h) Overlay of back scatter ICD image (greyscale) over the color-image of g. Bars: 50 μm (a) and 2 μm (b–h). Maps of other elementals are available as Fig.S1.
Multi-color EM by element guided identification of cells, organelles and molecules

Results

Large-scale EM of standard prepared rat pancreas fixed with aldehydes and osmium, embedded in epon (Fig. 1a; full resolution at www.nanotomy.org) was recorded using scanning transmission EM\textsuperscript{17, 18}. An endocrine area with three different cell types was selected (Fig. 1b). Traditional visual grey-scale analysis presumptively identifies these as a somatostatin-producing delta cell (top middle), a glucagon-producing alpha cell (left) and an insulin-producing beta cell (right). EDX analysis reveals informative maps of nitrogen, phosphor, sulphur and osmium (N, P, S, Os respectively) localization (Fig. 1c–f; and Fig. 1g,h for overlays; see Fig. S1 for more elemental results). N is abundant in all granules, as expected for highly concentrated peptides, irrespective of presumed cell identity. S is most abundant in insulin granules, as expected from the high cysteine content. The glucagon granules are found to stand out in the P map, whereas somatostatin shows neither pronounced S or P and is thus recognized on the sole presence of N. These compositional differences between granules are also revealed in qualitative comparison of the full EDX spectra (Fig. S4) and are furthermore reproduced using alternative, osmium-free sample preparation (Fig. S5). P maps also show condensed heterochromatin in the nucleus and the very dense endoplasmic reticulum network of the exocrine cells where P-rich RNA is translated. At high resolution also mitochondria are in the P map, which may reflect the abundance of phospholipids and ATP production. Overlay of the N, P, and S maps (Fig. 1g) clearly discriminates, in color indicating their elemental fingerprint, the separate granules. Not surprisingly, addition of the Os map, used as fixative and the only EM contrasting agent, adds the electron density determined by back scattered electron detection (BSD) from the EM image (Fig. 1h).

High-resolution definition of targets by EDX can further aid in molecular identification or assist in validation when applied to the recognition of elements specifically deposited at biomolecules of interest using antibody labeling. We choose a 1H6 monoclonal antibody that was raised against guanine quadruplexes (G4)\textsuperscript{19} and an anti-insulin antibody\textsuperscript{18}, for which we previously established immunolabeling on epon sections\textsuperscript{18}. Primary antibodies were subsequently labeled with secondary antibodies conjugated to gold (Au) and Cadmium-Selenide (CdSe)-based quantum dots (QDs), elements that are (nearly) absent in mammals\textsuperscript{20}. Nanotomy was performed (Fig. 2a; www.nanotomy.org) and an area was selected for EDX analysis (Fig. 2b). Shown is part of an insulin-producing beta cell, with in the top left a nucleus with euchromatin (white, light grey) and darker heterochromatin, especially present near the nuclear envelope. The 1H6 antibody developed against G4 structures shows strong reactivity in areas of heterochromatin\textsuperscript{19}, as also can be deducted from the electron-dense gold particles. In the cytosol, the presence of mitochondria and insulin-granules is clearly seen, the latter decorated with the electron-dense quantum dots (Fig. 2b). EDX analysis for Cd-based QDs, S-enriched insulin, Au (G4) and P-rich heterochromatin (Fig. 2c–f respectively; Fig. S4 for full elemental results) reveals that both Au and QDs are readily detected by EDX. Thus unambiguous identification of targets in biosamples by elemental analysis can be performed in addition to analysis by grey levels, size, or shape\textsuperscript{21, 22}. Note the high signal to noise ratio and co-localization of Cd (QDs; green) with S (insulin; blue), but not Au (1H6 antibody raised against G4; red) in the overlay (Fig. 2g). Similarly, Au (G4, red) localizes to P-rich areas in the nucleus (heterochromatin, blue; see also spectral data in Fig. S5), whereas the Cd (QDs, green) signal is enclosed within P rings (blue) that likely represent phospholipid membranes of the vesicles (Fig. 2h). Thus, EDX analysis allows for high resolution identification of targets in conjunction to endogenous elemental composition in mammalian tissues.
Figure 2 | Immuno-based identification of peptides and G4-DNA structures and endogenous elements. (a) Section of an islet immuno-labeled for G4 (10 nm immunogold) and insulin (QD655). The white area in the middle is an electron-beam pre-exposure artefact, but not hampering data analysis. Full resolution is available via nanotomy.org. (b) Zooming into the data reveals gold particles in the nucleus (upper left part) and quantum dots in insulin granules. Labels are identified based on grey levels and morphology. (c–f) Elemental content in the ROI as indicated. (g,h) Overlay of Au (red), Cd (green) and S (blue; g) or P (blue; h) allows identification of G4 structures based on gold presence and insulin granules based on Cd content. Note the localization of Cd on S-enriched insulin granules (g) and localization of Au to heterochromatin regions enriched in P in the nucleus (h). Bars: 50 μm (a) and 2 μm (b–h). See for more elements Fig. S2 and spectral analysis Fig. S6.
To explore EDX for biomedical microscopy, we used embedded material from our previous studies, namely a diabetic prone rat. 90% of these rats spontaneously develop diabetes, with the blood sugar level as an indication for the diabetic state. This experimental animal had not developed diabetes, and showed no signs of insulitis (Figs 1a, 2a, 3a and dataset). Unexpectedly, typical distinct vesicles within one cell are present at multiple locations. These multi-vesicle containing cells are at the border of the endocrine Islet of Langerhans and the surrounding exocrine tissue, the latter readily identifiable by the abundant endoplasmic reticulum and large zymogen vesicles (Fig. 3b, top). The adjacent cell has all characteristics of an endocrine cell (Fig. 3b, bottom). Note the presence of the small granules with a halo (typical for insulin) and other small granules showing an overall high electron density (typical for glucagon), but also large vesicles that resemble the zymogen granules in the exocrine tissue. Using the elemental characteristics to discriminate granules (Fig. 1), we analyzed the distribution of N, P and S (Fig. 3c–e; full elemental results in Fig. S3). Three distinct granules with characteristics of zymogen (N; red), glucagon (N in red and P in green, yellow appearance of coincidence) and insulin (N in red and S in blue, purple appearance) are present (Fig. 3f), which is more prominent at higher zoom (Fig. 3g). Color-coding of the backscatter image in green, a signal mainly caused by Os, allows to superpose the signal created by Os enriched in membranes (Fig. 3h). Interpretation of the multi-hormone and zymogen containing area is modelled on the STEM image (Fig. 3i). Thus, only with EM three distinct granules can be conclusively identified within the same cell and EDX shows the different composition based on different elemental ratios, thus without prior knowledge or anticipated labeling. Based on these new observations, we decided to substantiate our notion probing protein content, and subsequent optimization of double-immunolabeling confirmed our findings (Fig. 4)

Discussion

EDX imaging strongly aids in analysis of EM-data of mammalian tissue. Compared to previous approaches using EDX-imaging, which to our knowledge, have mostly been done on cryosamples, freeze dried samples and/or unstained epon we used chemical fixation, staining, and dehydration. Nanotomy allows analysis of complete cross sections of Islets of Langerhans, although the use of labels to detect proteins in an immunobased manner may benefit from Tokuyasu-sample preparation, which will be better compatible with epitope recognition, but this is typically performed on smaller sections (reviewed in refs ). Together with EM-based enhancements (silver) and immuno-labeling nanoparticles this enables recognition of multiple targets at the EM resolution level.

The use of high-sensitivity silicon drift detectors (SDDs) with high-current, high-resolution SEMs now allows determination of variations in elemental composition and elemental fingerprinting from the subcellular to the nanoscale level. These may be due to natural or enriched variations of endogenous elements, introduced stains (like osmium), and/or specific labels as shown for immunotargeted gold and QDs. Elemental detection has been explored before, mostly EELS focusing on quantitative determination of concentrations of typically one or two elements at the (sub)cellular level (reviewed in refs ). The Leapman lab pioneered EELS, which is capable of discriminating insulin and glucagon in cells. An early study on the use of QDs for immunolabeling directly noted the possibilities of elemental detection (EELS), posing that the diversity may be increased by using other elements in nanoparticles.
Figure 3 | EDX analysis reveals single cells that contain exocrine zymogen granules together with endocrine glucagon and insulin. (a) Overview as in Fig. 1a. (b) Area indicated in (a) by the cyan dot with parts of two cells with different granules based on grey levels and morphology. (c–e) Elemental maps as indicated. (f) Overlay of N (red), P (green) and S (blue) allows identification of cells and granules. (g) Zoom of the center part of f, note the small purple and yellow granules, as well as the big red granule, all present in the same cell. (h) Same region, with P and S combined with the backscatter image, which was reverted to red. Note the membranes around the vesicles. (i) Interpretation of the EDX data on the large scale STEM image showing the presence of three distinct granules in an endocrine cell. Bars: 50 µm (a), 2 µm (b–f) and 0.2 µm (g–i). See for more elements Fig.S3.
Implementation of EDX to discriminate ultrastructural features in the rat model for Type 1 diabetes (T1D) revealed distinct granules, believed to be cell-specific, within the same cells (Fig. 3), which have been substantiated by immunolabeling (Fig. 4). Such diversity has been reported in artificial differentiation protocols. T1D is caused by loss of the insulin-producing beta cells due to an auto-immune reaction of which the trigger is not known. More recently, the exocrine pancreas has been proposed to play a role in the destruction of beta cells, either by enhanced infiltration of immune cells in an animal model or by the notion that T1D patients have a 30% reduction in pancreas weight. Our observation may also hint to a malfunctioning of differentiation, or may reflect an exocrine/endocrine cell interaction, which opens the intriguing possibility that exocrine cells may interfere with beta cells. Thus the existence of mixed cells is readily identified by EDX. Although we only observed this in islets of two diabetic rats (total of 32 cells), not in two controls, a conclusive statement whether or not this phenomenon relates to a (pre)diabetic state should await results from a follow-up study. This will include examining human pancreas, but is beyond the scope of this paper.

EDX has been used to discriminate healthy or diseased cells based on elemental expression, or to address whether certain elements are enriched. Recently in an isolated cell model the potential to use elemental analysis and subcellular features was shown. Combination of EDX with EELS to explore additional elements with weak X-ray signals may thus further increase the elemental palette. EDX can be implemented in an existing TEM or SEM, is compatible with epon-embedded material, straightforward, and compared to EELS analysis, does not need a priori selection of imaging windows. Another mode of color-EM, CLEM, is widely used to localize targets typically between 0.05–1 μm. We foresee an integrated approach, where EDX and CLEM not only complement, but also allow crossing scales from rapid fluorescence screening of large fields-of-view to lower-throughput high-resolution elemental painting. Here we focused on qualitative measurement of chemically fixed targets, using protocols we – and others - typically use for EM examination of human tissues for diagnosis. Implementing other existing sample preparation protocols refined for research, like high pressure freezing followed by freeze substitution, may help to use EDX imaging in quantitative analysis and also allow determination of certain ions at high resolution in biosamples. Broad application of EM-EDX will allow experimental analysis on large-scale tissues using endogenous elements, multiple stains, and multiple markers and thus brings nanometer-scale ‘color-EM’ as a tool to unravel molecular (mis)regulation in biomedicine.
Figure 4  | Distinct granules in the same cell determined by immunolabeling. (a) Double immunolabeling of amylase (zymogen granules) with 10 nm immunogold and insulin with QD655 shows the appearance of distinct granules in the same cell based on morphology and electron density of the nanoparticles; immunogold is rounder and darker compared to the more rectangular and less electron dense QDs. Note that also unlabeled glucagon granules are present in the same cell. (b) Semi-automated nanoparticle annotation using the Fiji macro “golddigger” shows that distinct granules in the same cell occurs specifically in the islet (right) with the co-appearance of amylase (nanogold, blue) and insulin (QD655, green) labeling, and solely amylase (blue) labeling in the exocrine pancreas (left). Bars: 1 μm (a) and 5 μm (b).

Methods
Tissue and sample preparation

Embedded tissue blocs in EPON (Serva) were used as described before. Alternatively, in the case of osmium-free EPON embedding, tissue was post-fixed with 1% tannic acid (BDH chemicals, UK) in 0.05 M maleate buffer pH 5.15 for 40 minutes at room temperature. Furthermore, for additional membrane fixation, tissue was incubated in 1% poly-phenylenediamine (PPD, Merck-Millipore, Germany) in 70% ethanol for 20 minutes as one of steps during dehydration via graded ethanol series. All methods were carried out in accordance with relevant guidelines and regulations and the university ethical board for animal studies from the University Medical Center Groningen (UMCG), The Netherlands approved all animal experiments reported in this study. Ultrathin sections (100 nm) were cut and collected on formvar single slot pyrolytic carbon grids (EMS, Hatfield, Pennsylvania).

Post-embedding immunolabeling

Grids were incubated with tissue facing down on droplets on parafilm at RT. First, samples were etched with 1% periodic acid (Merck, New Jersey) in milliQ water for 10 minutes to increase antigenicity, and rinsed in milliQ (4 × 5 min). This was followed by 30 minutes blocking with 1% bovine serum albumin (BSA; Sanquin, The Netherlands) in tris-buffered saline (TBS), pH 7.4. For insulin and amylase labeling an alternative blocking buffer (1% BSA-c (Aurion, Netherlands), 1% cold water fish skin gelatin (Sigma-Aldrich), 35 mM lysine (Fluka), and 1% normal goat serum (Jackson Immunoresearch, UK) in TBS, pH 7.4) was used for both blocking and antibody dilutions. Next, anti-insulin (guinea pig; 1:1000 in 1% BSA/TBS, Invitrogen) combined with either monoclonal 1H6
(1 μg/ml in 1% BSA/TBS) or anti-amylase (rabbit; 1:50 in blocking buffer, Sigma-Aldrich), or anti-glucagon (rabbit; 1:50 in 1% BSA/TBS, Thermo Fisher) alone incubated for 2 hours, rinsed in TBS (4 × 5 min), followed by incubation for 1 hour with biotinylated secondary antibody (donkey-anti-guinea pig; 1:400 in 1% BSA/TBS, Jackson Immunoresearch) and subsequent rinsed in TBS (4 × 5 min). Finally, streptavidin-conjugated QD655 (1:1000 in 1% BSA/TBS; Life technologies, California) and secondary goat-anti-mouse antibody for 1H6 or goat-anti-rabbit for amylase conjugated to 10 nm gold (1:100 in 1% BSA/TBS; BBI solutions, United Kingdom), or goat-anti-rabbit-conjugated QD655 (1:1000 in 1% BSA/TBS; Life technologies) for glucagon were added for 1 hour and subsequently rinsed in TBS (4 × 5 min) and 0.1 M sodium cacodylate (2 × 5 min).

**Microscopy**

*Large-scale STEM (nanotomy)* - A large area scan using Scanning Transmission Detection (STEM) was made using a Zeiss supra55 SEM with Atlas as described before. From this dataset smaller areas covering several cell types were selected for EDX analysis. Pre-exposure to stabilize samples was carried out at low magnification, depending on sample area. Typically, for the complete islets, 80 kV was used for 1 hour in a TEM. We note that after careful pre-exposure, we analyzed samples without signs of electron-beam induced damage or carbon deposition visible when comparing SEM images obtained before and after inspection, even after EDX maps acquisition times exceeding 1.5 hours, i.e., pixel exposure times >1 ms (excluding time for drift correction procedures), and after spectral point acquisition times of ~30 s.

**EDX-analysis** - The experiments were performed on a FEI Verios SEM equipped with an Oxford Instruments X-Max 80 mm² Solid State EDX detector. As stated by the supplier, when the detector is fully inserted, the distance between the sample and the EDX window is 35.81 mm for a working distance of 5 mm and a tilt angle of 35°. Hence, the collection angle is 0.061 sr. For practical reasons the position might not be identical, since we optimized the position for collecting the maximum number of X-ray counts at 4 mm working distance. It is difficult to measure the exact distance between the window and the sample. The EDX detector has supplier-stated energy resolution of ≤ 127 eV at the Mn Kα line, ≤64 eV at the F Kα line, and ≤56 eV at C Kα, all at count rates of 20,000 cps. Peak position and resolution on the Mn Kα do not change more than 1 eV between 1,000 and 100,000 cps as calibrated upon installation. Au nanoparticles with a mean diameter of 2.7 nm dispersed on a thin Carbon support grid were detected in EDX, indicating that for high-density probes spatial resolution in EDX can approach the SEM probe size.

The imaging of the region of interest is performed using the ICD (In Column Detector) available in the Verios SEM at a resolution of 3072 × 2207 pixels, with dwell time of 30 us per pixel. All the regions of interest were imaged at 15 kV acceleration voltage and 26 nA current, at 4 mm working distance, in Ultra High Resolution (UHR) mode. The EDX maps were all taken at 15 kV primary beam energy and 26 nA current, at a working distance of 4 mm, in UHR mode. The Oxford Instrument AZtec software was used for the acquisition of EDX maps and point & ID analysis. The resolution of the maps is 2048*1408 pixels, with dwell time 20 us per pixel. The process time, i.e. the time over which the voltage signal generated by the detector is averaged, was set to 4 (on a scale from 1 to 6) in order to optimize the acquisition rate and to reduce the voltage noise, and we selected 2048 channels, that gives an energy window of 10 eV per channel. In case of point & ID analysis, 4096 channels were used, with an energy of 5 eV per channel. Because of the long acquisition time for the EDX maps, sample and stage drift plays an important role. For that reason, the AZtech software drift correction option was activated, meaning that after a certain amount of time, determined by the software based on the drift speed, an EM image is acquired, which is compared to an original image taken at the start of the EDX map. The software automatically detects
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particles or shapes in the image, from which it calculates the drift and compensates for it. The lifetime, i.e. the effective time in which the spectra is acquired and integrated, and the collected number of frames for each field of view are listed in Supplementary Table S1.

Image processing

In Fiji (http://fiji.sc/), three selected elements were merged as RGB. All image processing has been applied to the whole image in a linear fashion using Fiji and/or Adobe Photoshop. To discriminate 10 nm immunogold and QD655 nanoparticles upon immunolabeling of amylase and insulin, semi-automated annotation using the Fiji macro “golddigger” was performed.

Acknowledgments

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Multi-color EM by element guided identification of cells, organelles and molecules

References


Supplementary information

Figure S1 | Additional element-specific images as indicated. See Fig. 1 for details.
Figure S2 | Additional element-specific images as indicated. See Fig. 2 for details.
Figure S3 | Additional element-specific images as indicated. See Fig. 3 for details.
Figure S4 | EDX spectral differences between different hormone-containing granules. (a) Same area as in manuscript Figure 1 with circles indicating the granules for which EDX spectra are shown in (b). (b) EDX spectra for the respective granules in (a). Drawn lines denote spectra obtained by averaging the data collected in the spatial maps over the indicated granules. Dashed lines indicate spectra obtained by 30 second integration on a single spot within the granule. All spectra are normalized to the C peak and smoothed using a standard Matlab routine. All spectral features in the spectra obtained from the EDX maps are qualitatively reproduced in the spot integration curves, confirming the validity of the acquisition time for the EDX maps. The pronounced Al signal comes from sampleholder, probably from back-scattered electrons generated in the sample that hit the sample holder. (c) Comparison of EDX spectra obtained for the three different granules, revealing the increased level of S in insulin granules, P in glucagon granules, and relative absence of both for somatostatin. Note also the pronounced differences in Os staining level corresponding to the granule interpretation in standard grey-scale EM. Scalebar in (a) 2μm. Experimental settings: 15kV beam energy at 26nA, EDX: process time 4, 4096 channels, 5eV per channel.
Figure S5 | EDX data on glucagon-producing alpha cell using an Osmium-free sample preparation. Quantum dots are labelling glucagon to allow identification of the glucagon granules (a) SEM image of an alpha cell. (b) Magnified image of the boxed area in (a) with clear visibility of QDs on the granules. (c) EDX spectra obtained by integrating the spectral maps over the glucagon granule and the reference area indicated in (b). (d, e, f) EDX maps of the area in (b) depicting the signal for the indicated spectral lines for (d) P, (e) S, and (f) N. Note how the (speckled) S signal follows the distribution of the ZnS-shelled quantum dots, visible especially in the upper right corner. The glucagon granules are clearly discerned in the P map. Sample preparation was similar to that for the data in the manuscript, except for the omission of Osmium and a section thickness of 300 nm. The spectra in (c) have been normalized to the C peak and smoothed using Matlab. Scale bars are 5 µm in (a) and 500 nm in the other panels.
**Figure S6 | EDX spectra comparison for quantum dot and gold nanoparticle labelled sample.**

(Manuscript Fig. 2). (a) Same area as in Fig 2b with circles indicating regions over which EDX spectra were averaged for (b). Inset is shown for the area indicated with yellow rectangle. (b) EDX spectra averaged over the indicated areas. Au signal (targeted to 1H6) is clearly visible in conjunction with a strong P peak from heterochromatin. In the green area, Cd is clearly present originating from the quantum dots core, together with weaker Zn from the quantum dot shell. Note that the S peak appears slightly more pronounced compared to, e.g., Os and Cl than in Fig. 1b, presumably due to the additional presence of the ZnS quantum dots shell. Experimental settings as stated in the manuscript, spectra normalized to the C peak and smoothed using Matlab. Scale bar 500nm, and 100nm in the inset.
Table S1 | EDX acquisition characteristics per FOV

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