Nanoparticle discrimination based on wavelength and lifetime-multiplexed cathodoluminescence microscopy

Garming, Mathijs W H; Weppelman, I Gerward C; de Boer, Pascal; Martínez, Felipe Perona; Schirhagl, Romana; Hoogenboom, Jacob P; Moerland, Robert J

Published in:
Nanoscale

DOI:
10.1039/c7nr00927e

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 15-04-2019
Nanoparticle discrimination based on wavelength and lifetime-multiplexed cathodoluminescence microscopy

Mathijs W. H. Garming,a I. Gerward C. Weppelman,a Pascal de Boer,b Felipe Perona Martinez,c Romana Schirhagl,d Jacob P. Hoogenboomd,e,a and Robert J. Moerlandb,a

Nanomaterials can be identified in high-resolution electron microscopy images using spectrally-selective cathodoluminescence. Capabilities for multiplex detection can however be limited, e.g., due to spectral overlap or availability of filters. Also, the available photon flux may be limited due to degradation under electron irradiation. Here, we demonstrate single-pass cathodoluminescence-lifetime based discrimination of different nanoparticles, using a pulsed electron beam. We also show that cathodoluminescence lifetime is a robust parameter even when the nanoparticle cathodoluminescence intensity decays over an order of magnitude. We create lifetime maps, where the lifetime of the cathodoluminescence emission is correlated with the emission intensity and secondary-electron images. The consistency of lifetime-based discrimination is verified by also correlating the emission wavelength and the lifetime of nanoparticles. Our results show how cathodoluminescence lifetime provides an additional channel of information in electron microscopy.

Introduction

High-resolution identification of materials in electron microscopy images is a challenge throughout many areas of science. The detection of visible photons emitted resulting from electron irradiation, so-called cathodoluminescence (CL), provides a straightforward analytical alternative to the electron signals. Typically, spectrally selective CL detection is used to identify materials with different composition, especially in materials and geosciences.2–8 Recently, CL microscopy is gaining interest also in the biological, optical, and nano-sciences as electron beam excitation provides a natural solution to circumvent the diffraction limit of photon-excited fluorescence microscopy.9–15 In addition, compared to correlated fluorescence and electron microscopy (CLEM),16 detection of CL may alleviate the need to register images obtained on different microscopes, i.e., light- versus electron-based.17 A major challenge in CL microscopy, especially in the biological sciences, is the rapid degradation of CL signals, particularly from organic molecules, under electron irradiation.18 Cathodoluminescent nanoparticles (CNPs), for example phosphors composed of metal oxides13,19–21 and fluorescent nanodiamonds (FNDs),22 are an attractive alternative for labeling in biological sciences with size and thus resolution that can go down to a few nanometers.23–25 Multicolor experiments can be realized by employing various types of emitters, such as rare earth ions15 and FNDs with engineered defects to modify the emission spectrum.22 However, as in fluorescence microscopy, the analytical power of spectral discrimination is inherently limited due to overlap of emission bands and availability of filters.

In fluorescence microscopy, lifetime imaging26,27 provides additional or otherwise unobtainable data, on, for example, the local (optical) environment26–30 or state of fluorophores.31 Moreover, lifetime imaging can be used to differentiate between various emitters.26 With strong CL signals, the fluorescence lifetime can be obtained by fitting exponential curves to decay curves after excitation with continuous beams.32,33 For low signals, single photon counting can still accurately resolve lifetimes by determining, e.g., antibunching curves34 or photon arrival time histograms.35 The latter option can be used to minimize electron-induced damage to the sample by exciting the sample with short electron pulses, while also maintaining the native resolution of the Scanning EM (SEM).16,37

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26

27

28

29

30

31

32

33

34

35

36

37
Here, we demonstrate the ability to discriminate between various CNPs in the time domain. We show that we can measure the emission intensity of CNPs while exciting them with a pulsed electron beam, which is generated by a commercial beam blanker.\textsuperscript{37} For each pixel in the intensity map we measure the intrinsic lifetime associated with the type of emitter at that particular location. To confirm our discrimination based on the time-domain data, we measure spectrally-selective CL intensity maps for comparison.

Results & discussion

We have modified a standard electron microscope to include a high-NA optical objective, which is part of an inverted optical microscope,\textsuperscript{14,38,39} and have added a pulsed electron-beam mode suitable for time-resolved imaging (see Fig. 1). With this setup, we obtained electron pulses with lengths down to 80–90 ps. We employ a Time-Correlated Single-Photon Counting (TCSPC) scheme (PicoQuant PicoHarp 300), which is often used in fluorescence microscopy to determine the fluorescence lifetime of fluorophores. In that case, the TCSPC clock signal is typically issued by a pulsed laser. Here we drive the blanker with a pulse generator and use the same signal to synchronize with the TCSPC hardware.

Samples with dispersed CNPs are obtained by spincoating from solution. In order to establish the distribution of lifetimes of each type of nanoparticle, we first prepare two samples, where each sample contains only one type of particle. Here, we use cerium-doped Lu\textsubscript{3}Al\textsubscript{5}O\textsubscript{12} (LuAG:Ce) particles (Boston Applied Technology, Inc.) that exhibit a transition from the 5d level to the 4f ground state\textsuperscript{40} of Ce\textsuperscript{3+}, and FNDs (Adamas) that exhibit a transition from the 2A state to the 2E state,\textsuperscript{41} associated with the display of CL by primarily the neutral NV\textsuperscript{0} center.\textsuperscript{42} Typical photon arrival time histograms are shown in Fig. 2a. We approximate the measured arrival times with a single exponential, which yields a typical decay time of the CNP. We collect the decay time of 28 FNDs and 46 LuAG:Ce CNPs and determine the distribution of lifetimes (Fig. 2b). The difference in lifetime distribution between the two particles is clearly visible; LuAG:Ce nanoparticles have a typical lifetime of about 50 ns, with a width of 14 ns (2\(\sigma\)). The
FNDs on the other hand have a substantially shorter lifetime of typically 30 ns, with a width of 10 ns (2σ). Finally, in Fig. 2c, the measured emission spectra of both types of CNPs are shown.

**Stability of lifetime under e-beam irradiation**

Next, we investigated the stability of the lifetime measurements over the irradiation time. In contrast to intensity, which changes over time during the irradiation with charged particles, lifetime is a relatively stable quantity. This is highlighted in Fig. 3a–d. Fig. 3a displays the measured lifetime of three FNDs over a time span of 10 minutes. The photon arrival histograms were determined every 5 seconds. The number of photons in every histogram is displayed in Fig. 3b over the same time span. Similar measurements for the LuAG:Ce particles are shown in Fig. 3c and d. Despite the fact that the CL intensity varies (likely due to bleaching and drift of the electron beam), the measured lifetime remains extremely robust. As a particularly extreme example, the lifetime of the FND displayed with a blue curve in Fig. 3a varies between a maximum of 27 ns and a minimum of 20 ns and has a mean of 25.0 ns ± 12% (3σ). This happens while the intensity drops by a factor of 29. Whereas bleaching of FNDs has been reported in literature, the exact mechanism is still subject of research. Potentially the creation or annihilation of surface charge traps, dynamics of impurities or charge carriers in the vicinity of the vacancy centers, or quenching of nitrogen-vacancy centers near the surface by a thin carbon contamination layer grown by e-beam-induced deposition play a role. This is subject to further investigation.

**Cathodoluminescence lifetime maps**

Subsequently, a sample is prepared that contains both types of CNPs, with approximately a 1 : 1 ratio of the number of particles of each type. A secondary-electron (SE) image of a 6 × 6 μm region on the sample is displayed in Fig. 4a. We scan the same area again, but now with a pulsed electron beam at a step size of 100 nm and a dwell time of 0.5 seconds. For every pixel, the photon arrival histogram is determined, which is then fitted with a single exponential decay. Thus, we simul-

![Fig. 3](image_url)

**Fig. 3** Stability of lifetime and intensity versus irradiation with the electron beam, for three particles of each type (LuAG:Ce and FND). Lifetime versus e-beam irradiation duration, determined every 5 seconds, of three FNDs and LuAG:Ce particles is shown in panel (a) and (c), respectively. Panels (b) and (d) show the corresponding CL intensity during the measurement. Despite (severe) differences in intensity, the lifetime of the nanoparticles is a robust parameter during the measurement.
Simultaneously measure the intensity and lifetime of the emitters on the sample. The intensity of both type of particles can differ greatly, which on a linear scale could render the less intense particles invisible. Therefore, we show the CL intensity map in Fig. 4b on a logarithmic scale. Note that this leads to an apparently larger size of the particle. The correlation between the CL intensity and the SE image can be seen in Fig. 4c, where the intensity is shown in a false color scale for identification purposes only.

The lifetime map that we obtain from the fitting procedure is shown in Fig. 5a. Here, the lifetime map is thresholded with the intensity, such that areas without CNPs do not show as noise in the lifetime maps. Clearly, there are two dominant lifetimes in Fig. 5a, corresponding to the distribution of lifetimes as measured before and displayed in Fig. 2b. Particles with a lifetime of around 20–30 ns correspond to FNDs, and particles with a lifetime of about 50–70 ns correspond to LuAG:Ce particles. For comparison, the scans were repeated, inserting optical filters to discriminate between the two particle types: a short pass filter with an edge wavelength of 550 nm in order to only see LuAG:Ce particles, and a long pass filter in order to see the emission by the FNDs. Due to the broad spectrum and high CL intensity, the LuAG:Ce particles are suppressed in intensity but visible as well. The results for the short pass filter are shown in Fig. 5b, whereas the results for the long pass filter are shown in Fig. 5c. As an example, the low-lifetime FNDs highlighted by a white square in Fig. 5a clearly only show up in the longer-wavelength emission channel in Fig. 5c, corresponding to the bulk of the emission spectrum of FNDs. Thus, the spectral data confirm the validity of our CL lifetime-based discrimination.

For higher resolution CL lifetime imaging, the step size of the electron beam can be reduced. In Fig. 6 we show three different nanoparticles scanned with a step size of 50 nm. As the current with pulsed electron beam operation is reduced, image acquisition times are longer compared to continuous...
operation and thus choice of step size is a balance between resolution required for particle identification and field of view size. The results shown in Fig. 6 confirm the above conclusion that we can discriminate the particles based on lifetime, where here we are limited by the particle size.

CL lifetime in stained biological sections

Application of CL lifetime imaging for nanostructural characterization may range from geosciences and materials sciences, phosphor particle detector development, to visualization of labels in biological sciences. As an application example, we here demonstrate that CL lifetime imaging can be conducted on biological sections after fixation, staining, and sectioning protocols. Fig. 7a shows a back-scattered electron (BSE) image of a 300 nm section of macrophages incubated with the FNDs. The image shows good ultrastructural preservation and fine intracellular details are well resolved. The FNDs are however not visible in the BSE images. In Fig. 7b and c we show a zoom of an area where CL was detected, presumably from uptaken FNDs. The CL lifetime map in Fig. 7e indeed unequivocally
identifies the FNDs as being the source of the CL emission log-
arithmically plotted in Fig. 7d (cf. Fig. 2b). The weaker CL
signal for one of the three FNDs may be due to an FND buried
deeper within the 300 nm section. Indeed, while the other two
particles stood out in the SE image as well (data not shown),
as expected for surface-exposed FNDs,\(^{25}\) no SE signal above
background tissue signal was recorded at this location. The
BSE-CL overlay allows for high-resolution localization of the
CNPs in the macrophage ultrastructural environment and life-
time data is useful to identify these as FNDs.

Conclusions

We have shown that lifetime-based imaging and particle dis-
crimination is a stable modality in electron microscopy, useful
for analytical and correlative imaging purposes. Based on lif-
time, we demonstrated discrimination of particle type in a
mixture of two CNPs dispersed over a surface, and CL lifetime
imaging of CNPs in tissue sections. The lifetime of the CNPs
measurements show very good robustness over time, with only
mild variations even when the intensity itself diminishes sig-
nificantly.

For applications, we believe that correlative SE and CL life-
time imaging is suitable for high-resolution characterization
and imaging throughout the nanosciences. Particular areas of
interest may include materials sciences and geosciences in
general, and characterization of the homogeneity of phosphors
for use in particle detectors or of nanoparticle assemblies in
particular. In addition, the pulsed electron excitation paired
with the possibility to obtain accurate lifetime estimates from
histograms with low photon counts\(^{35}\) may be particularly ben-
eficial for systems suffering from electron-beam degradation,
such as labels in biological tissue sections. Moreover, instead
of or combined with multi-color CL imaging, the lifetime of
the nanoparticles may be used to increase the number of
labels discernible in EM; e.g., green-fluorescing nanodiamonds
and LuAG:Ce have very similar optical emission spectra but
have different lifetimes,\(^{39}\) which is sufficient to distinguish the
particles. Time-resolved CL imaging can also be beneficial in
combination with other characterization techniques such as
energy-dispersive X-ray spectroscopy,\(^{50}\) e.g., to distinguish
materials with similar elemental composition such as different
(carbon-based) FNDs and native biological tissue or polymeric
materials. Finally, CL lifetime microscopy may be used to
recover more information about the local environment of the
nanoparticle, such as structures which modify the optical local
density of states,\(^{37,51}\) or the actual (surface) structure or doping
concentration of the nanoparticle itself.\(^{52–54}\)

Methods

Particle samples

Sample preparation. The sample containing both types of
CNPs was prepared by a combination of spincoating and drop-
casting particles in solution on an indium-tin oxide (ITO)
covered glass slide. The Ce doped LuAG particles were dis-
persed in ethanol at a concentration of 2 mg ml\(^{-1}\) and the
solution was spincoated on the ITO at 1000 rpm for 2 minutes.
To ensure sufficiently dense coverage, the spincoating was
done five times consecutively. The DI water based solution in
which the FNDs are supplied was diluted by a factor 20 for a
concentration of approximately 0.05 mg ml\(^{-1}\). A 10 μl droplet
was deposited on the LuAG coated ITO slide and left to dry on
a hot plate at about 50 °C. Sample containing only a single
particle variety were prepared in the same manner, omitting
deposition of the other CNP type.

SE and CL measurements. We used a beam energy of 4 kV
in conjugated blanking mode\(^{17}\) to excite the CNPs and
measure SEs. The current in the continuous electron beam
was 19 pA, and while in this mode we retrieved the SE image
of the sample surface. Then, we switched on the pulse genera-
tor to drive the beam blanker at a repetition rate of 500 kHz.
This results in two pulses per cycle; one at the rising edge of
the pulse and one at the falling edge. Therefore, a total
measurement time of 1 μs after each electron pulse is available
to build the photon arrival histogram. With longest decay
times of about 90 ns, this is sufficient to faithfully retrieve the
arrival histograms. An avalanche photodiode (PicoQuant) was
used to collect the photons emitted by the sample.

Macrophage samples

Sample preparation. For the uptake experiments, ground
HPHT diamonds (Adamas Nano; on average 120 nm hydro-
dynamic radius and 900 NV centers per particle as stated by
supplier) were used. The particles were coated with a protein
copolymer consisting of a C4 domain and a K12 domain. C4 is a
tetramer of a 98 amino acid long, hydrophilic random coil
polypeptide, which is rich in glycines, prolines and other
hydrophilic amino acids.\(^{55}\) K12 consists of 12 lysines which
are positively charged. The coating not only improves colloidal
stability but the positive charge also might favor formation of
NV0. The C4K12 protein polymers were obtained as described
elsewhere.\(^{55}\) In short, secreted expression by genetically
engineered *Pichia pastoris* strains was used. The protein was
synthesized with a so-called methanol fed-batch fermentation.
Then the protein-containing supernatant was separated from the
*P. pastoris* cells by centrifugation (30 min at 16 000g) and
subsequent microfiltration. The protein was purified by select-
tive precipitation with ammonium sulfate (at 45% saturation).
After a dialysis against 50 mM formic acid the protein was lypo-
philized and the protein powder was stored at room tempera-
ture. Before coating the polymer was diluted in MilliQ water to
a concentration of 7.36 mg ml\(^{-1}\). The protein was passed
through a 0.22 μm filter to remove aggregates.

The diamond particles were coated with the C4K12 protein
copolymer by mixing the stock solution of nanodiamonds
(100 μg ml\(^{-1}\) in water), with the protein solution followed by
incubating for 30 minutes. The final particles contain roughly
4 C4K12 molecules per 100 nm\(^2\) of nanodiamond surface.
All solutions were prepared under sterile conditions.
J774A.1 macrophages (LGC Standards, Germany) were cultured in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% FBS, 1% penicillin/streptomycin. Cells were incubated with 1 μg ml⁻¹ coated FND in cell culture medium for 5 hours at 37 °C and 5% CO₂. After removal of culture medium with FNDs, cells were fixed with 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 30 minutes at room temperature. After washing with 0.1 M cacodylate buffer, cells were incubated with 1% osmiumtetroxide/1.5% potassiumferrocyanide in 0.1 M cacodylate buffer for 30 minutes at 4 °C, 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 polymerised 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.

**BSE, SE and CL lifetime measurements.** Lifetime and CL measurements on FNDs incorporated in macrophages have been performed with an FEI Quanta FEG 200 SEM by scanning the sample with a pulsed electron beam with a step size of 50 nm and a dwell time of 1 s. The acceleration voltage and beam current were set to 3 kV and 13 pA, respectively. At every electron-beam position, the SE signal, CL intensity, and photon arrival time histograms were simultaneously recorded and lifetime values were obtained from single exponential fits to the histograms. As BSE detection provides better contrast on biological tissue than SE detection, and our time-resolved setup is not equipped with a BSE detector, the biological structure of the cell was imaged with an FEI Verios 460 SEM. By referring to the SE images obtained during the time-resolved measurement, the region of interest is retrieved, and SE and BSE images are simultaneously obtained with a 3 kV, 0.8 nA continuous electron beam.

**BSE and CL image overlay.** Both type of measurements described above, i.e., the retrieval of the BSE and CL lifetimes, also result in an SE image. Therefore, we used the SE images as ground truth data and registered the SE channels from both independent measurements onto each other. Here, we assumed an affine transformation. After the registration, the same transformation is used to map the BSE and CL images onto each other, resulting into Fig. 7c. The registration was performed with Imagej plugin together with the TurboReg plugin.

### Acknowledgements
This research was supported by the Netherlands Organization for Fundamental Research (NWO) and the Stichting voor Fundamenteel Onderzoek der Materie (FOM, Foundation for Fundamental Research on Matter) grant number 10PR2826. RS acknowledges support from NWO-FOM via grant number FOM-G-36, PdB from Stichting Technische Wetenschappen (STW) – Perspectief voor de Topsectoren – Microscopy Valley grant number 12718, project leader Ben Giepmans. FPM is supported by A CONICYT scholarship from the Chilean government. Part of the work has been performed in the UMC Microscopy and Imaging Center (UMIC), sponsored by ZonMW grant 91111.006 and NWO 175-010-2009-023.

### References

### Conflicts of interest
Our integrated microscope and cathodoluminescence detection set-up served as a prototype for a product by Delmic BV. JPH is co-founder and shareholder in Delmic.