nature portfolio

Peer Review File

Multicolor cathodoluminescence imaging of single lanthanide nanoparticles

Corresponding Author: Professor Maxim Prigozhin

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript submitted by Rehman et al. presents an investigation into single-particle cathodoluminescence (CL) imaging of small lanthanide nanoparticles, termed "cathodophores." A key challenge identified for multicolor CL imaging is the nonlocal CL excitations caused by secondary electrons. To overcome this issue, the authors utilized a customized spectrometer to selectively isolate characteristic wavelength windows, corresponding to specific dopants. By employing advanced data processing techniques, they were able to identify individual nanoparticle dopants even in densely packed samples. Furthermore, the authors experimentally validated a previously proposed linear relationship between nanoparticle diameter and CL photon detection rate, which is an expected but crucial result.

Overall, the manuscript is well written and provides a valuable contribution to the field. The detailed supporting information, especially the images of individual channels, is commendable.

I have a few suggestions that would further improve the manuscript:

1. Consider merging the figures related to nonlocal excitations into a single comprehensive figure (Figure 1). This would enhance clarity and allow for a more cohesive presentation of this important topic.

2. While the manuscript introduces multicolor CL imaging, it would be beneficial to clearly highlight the novelty and impact of this approach. Provide precise examples in both the Introduction and Discussion where this technique could be applied. Additionally, outline its advantages over existing ColorEM methods (incl. EDXS, and EELS (in STEM))

3. I encourage the authors to explicitly highlight their development that enabled multicolor CL imaging. From my current understanding of the discussion, it appears that minimizing energy deposition in neighboring nanoparticles was essential to achieving this. However, as presented, this outcome seems to stem more from fundamental physical principles rather than a novel experimental or technical advancement. Clarifying this distinction would help underscore the unique contributions of the work.

4. Additionally, the manuscript refers to a “comprehensive framework” at several points, but its precise nature remains unclear to me. Please provide a more detailed explanation of what this framework entails, particularly its role and significance in the context of the study.

Reviewer #2

(Remarks to the Author)
In this paper, the authors demonstrate single-particle multicolor CL imaging of lanthanide nanoparticles. Experiments show a scaling law between nanoparticle size and luminescence intensity, and Monte Carlo calculations support the scaling law. These results, while interesting, are predictable for researchers in this field, lack novelty, and are not worthy of publication in

Natrue Communications. For publication, we request additional experiments on 1 and 2 below and explanations for 3 and 4.

1. When applying this imaging technology to biological and materials science, there will be few situations where only lanthanide nanoparticles will be observed. There is a concern that sufficient spatial resolution and luminescence intensity may not be accomplished due to the influence of surrounding materials. The authors need to show single-particle multicolor CL imaging data for biological or materials science applications and discuss the luminescence properties of single lanthanide nanoparticles, including their spatial resolution, luminescence intensity, and CL background.

2. Regarding the above question, fluorescence imaging is also necessary for the areas observed by CL imaging. This is correlative imaging by CL and fluorescence.

3. The authors use the term secondary electrons in the paper, how did they prove this? Is it possible to ignore the possibility that e.g. reflected electrons or characteristic X-rays could have excited the surrounding lanthanide nanoparticles?

4. When using NaYF4:Dy, increasing the concentration of Dy causes concentration quenching, which lowers luminescence efficiency. Please explain the reason for using NaLnF4.(Ln = Lanthanide)

Reviewer #3

(Remarks to the Author)
The manuscript "Multicolor Cathodoluminescence from Single Lanthanide Nanoparticles" by S. A. Rehman addresses a critical limitation to achieving nanometer-scale multicolor cathodoluminescence (CL) imaging. It demonstrates that the resolution of the multicolor CL imaging can be improved to a few tens nanometer scale. The authors experimentally demonstrate a potential nonlocal excitation pathway by designing a sample with a concentrated cluster of particles on one side and sparsely distributed single particles on the other. Using carefully prepared samples that do not contain aggregated particles, the authors also demonstrate highly spatially-resolved multicolor imaging with a resolution of ~12 nm. Moreover, the CL-intensity curve as a function of particle diameter presents interesting results, showing the quantitative nature of CL from nanoparticles. The information in the manuscript will be of interest to the SEM and TEM-CL communities.
The author effectively demonstrates potential misinterpretations that can arise during CL multi-color imaging through well-structured experiments, and the size-dependent data is quite intriguing. However, sub-20 nm scale CL multicolor imaging using SEM and TEM has already been reported in other studies (Swearer, D. F., et. al., ACS Photonics 2021, 8, 1539-1547; Mauser, K. W., et. al., ACS Nano 2021, 15, 11385-11395).

I agree that the aggregation of LNPs is the main source of nonlocal excitation as presented in Figure 1. However, the evidence supporting the conclusion that secondary electrons are the cause of nonlocal excitation appears to be insufficient. There seems to be a great discrepancy in the length scale between the Monte Carlo simulation results and the distance-dependent intensity results shown in Figure 2. Therefore, the interpretation of the non-local excitation and the size-dependent results does not appear to be convincing, and thus the analysis does not seem to convey novel scientific insights.

Considering the above two aspects, unfortunately, this research does not seem to present a significant scientific advancement to meet the high standards required for publication in Nature Communications. I would recommend the authors to submit the work elsewhere once the following concerns and suggestions are properly addressed:

Major comments.
Q1-1. In Figure 3A, the authors show that less than 2% of the energy is deposited into an adjacent LNP that is 20 nm away from the particle excited by the electron beam. The energy absorption decays exponentially (or according to a quadratic function) with distance and becomes almost zero when the particles are separated by approximately 200 nm. This is three orders of magnitude shorter than the distance at which the Monte Carlo simulation shows almost zero probability of energy absorption. This points out that the secondary electrons from a single particle are unlikely to excite aggregated particles located 200 μm away, which
Also, although the LNP contains heavier elements than the Si substrate, the secondary-electron generation event and its absorption by aggregated particles would be more significant when the electron beam is focused on the Si substrate, which is near the aggregated particles, compared to a single particle 200 μm away from the aggregated particles. This suggests that secondary electrons may not be the primary source of nonlocal electron excitation again.
In this regard, the authors may consider distance-dependent Monte Carlo simulation results to check the number of secondary electrons generated on the Si substrate and absorbed by a single particle, similar to what is shown in Figure 3A?

Q1-2. If secondary electrons are not the source, energy transfer (Förster- or Dexter-type?) could be the origin of the nonlocal excitation. This hypothesis could be tested using a system similar to that in Figure 2, but with non-emissive particles that generate a similar number of secondary electrons as $NaHoF_4$, instead of $NaHoF_4$ itself. If the $NaDyF_4$ spectrum is no longer observed, it would suggest that the origin of the nonlocal excitation observed in single particles originated from energy transfer from the single particle to the aggregated particles.

Q2-1. The experimental observation of CL-intensity dependence on particle size, along with its verification through simulation results, is a fascinating finding that could significantly enhance the understanding of the quantitative nature of CL from nanoparticles in both the SEM CL and TEM CL communities. However, I'm unsure if we can confidently describe the relationship between radius and intensity in Figure 4A as linear. For example, in the first panel of Figure 4A, the intensity of the 20-nm particle is around 1, while for the 30-nm particle, it is 3. Could it be cubic (volume) dependence?

Q2-2. According to page 24 of the supporting information, this linearity breaks down around 15 nm when the electron beam energy is reduced. Have you experimentally observed this breakdown in linearity using an electron beam with an energy lower than 0.5 keV?

Q3. In some lanthanide-doped particles, the CL intensity may not increase linearly with the electron-dose rate, as excessive excitons and free carriers generated under high dose rates can alter the population distribution of emitting excited states. Were the measurements or simulations in the manuscript conducted with dose-rate dependence taken into account, or were they performed within the regime where intensity increases linearly with the electron-dose rate?

Minor comment.
Q1. In the first section, 'Nonlocal electron excitation can prohibit single-particle multicolor CL imaging', it is suggested that nonlocal electron excitation dominates multicolor CL imaging, hindering both quantitative and qualitative analysis of LNP emission. And, in the following section, minimizing the LNP aggregation mitigates nonlocal electron excitation. As far as I understand, the only difference between the experimental conditions in Figures 1 and 3 is the distribution of LNPs on the Si substrate, which determines the presence of nonlocal electron excitation. However, since only a single particle is shown in Figure 1, it is difficult to imagine that aggregated LNPs are nearby (within 200 μm). Could you provide a lower-magnification image of the sample in Figure 1?

Q2. Could you please define "CL brightness"? Is it different from the number of photons?

Reviewer #4

(Remarks to the Author)
What is the difference between "multi-color CL" and CL spectrum imaging? It seems unnecessary to rename an established technique.

It is not clear what value Figure 1b and 1c add to the discussion. Also, they are plotted on different y-axis scales. If they are necessary please specify why in the text and plot both on either the exponential scale or linear scale, not one on either.

It is clear from the spectra in Figure 1a that the Dy sample has an emission peak that overlaps with what is defined as the Ho channel. Without showing the spectra (not normalized) for the plots in 1d I am left wondering if the Ho map is just representing part of the Dy emission around 650nm.

Figure 2d does not seem to agree with the discussion in the text. The text says that a thick layer of NaDyF4 is separate from a sparse coverage of NaHoF4 particles. 2d shows a mix of particles, correct this to match the text discussion or state why it is different.

The sentence "Interestingly, since the LNPs were composed of heavier elements than the Si substrate, they produced more secondary electrons." needs revision if it is necessary. It is not "interesting" that higher Z elements emit more SEs, this is a fact included in any introductory discussion of SE generation.

This statment " The simulations showed that for two neighboring LNPs, if one was excited with the electron beam, <2% of the energy was deposited into the neighboring LNP, even if the two LNPs were in direct contact. Such minimal crosstalk indicated that multicolor CL imaging with nano-crystals should be possible in samples containing only individual non-aggregated LNPs" is quite concerning.

How can the authors argue that the Ho signal from the Dy particles is an artifact if the above statement is accurate? Figure 1d shows one particle in the field of view, not even "touching" another particle. Why would there be an issue with the emission from such a particle? clearly there is not as in Figure 3 the authors claim all is well and there are no artifacts present. The only "mitigation" that was presented seems to be a simulation and then preparation of a more sparse distribution of particles. Though even the latter part of that statement seems wrong as in Figure 3 the particles are close or touching but with no issues with artifacts.

In the conclusion section there is the statement "The key advancement that enabled localized CL excitation was overcoming nonlocal CL signal caused by stray secondary electrons. These stray electrons could excite nano-crystals hundreds of micrometers away from the point of beam-sample
interaction. " From the points described above I find this advancement to be misleading. The overcoming of nonlocal signal seems to be by making particles spaced farther apart (a logical and elementary solution) but then the authors show in Figure 3 that this doesn't matter... Yes, if you directly excite particle aggregates you can get signal from multiple particles as evidenced by the monte carlo simulations but why single particle analysis is an issue is not clearly presented in this manuscript.

Reviewer #5

(Remarks to the Author)
In the manuscript titled Multicolor Cathodoluminescence from Single Lanthanide Nanoparticles, Sohaib Abdul Rehman et al. performed the cathodoluminescence in lanthanide nanoparticles and present intriguing findings.

The results address the issue of nonlocal excitation in multicolor cathodoluminescence (CL) imaging, facilitating single-particle multicolor CL imaging. This study enhances both the resolution and accuracy of imaging while offering new insights into nano-research. However, minor revisions are needed before this manuscript can be accepted for publication in Nature Communications.

1．The abstract could benefit from being more concise.

2．The authors should include more information on recent advancements in optical excitation (photoluminescence, PL) compared to electron beam excitation (cathodoluminescence, CL) in the introduction. This will highlight the significance of the article's findings.

3．The data presented in Figure 3 are adequate to illustrate the impact of secondary electrons (SE); if possible, please include more detailed information.

4．Despite the low accelerating voltage of 3 kV used in the manuscript, some damage to the nanoparticles still occurs, affecting imaging accuracy. Please include the results of reproducibility experiments to address this issue.

5．The manuscript would benefit from proofreading by a native English speaker or a professional language editing service.

Reviewer #6

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have addressed the main concerns raised in the previous review round. In particular, they have conducted a sufficient number of additional experiments and provided new figures, which help to clarify the points in question.

The current version of the manuscript represents a meaningful improvement over the previous submission. I believe the manuscript is now acceptable for publication.

Reviewer #3

(Remarks to the Author)
I appreciate the authors' detailed responses and the additional simulations to address this question. Substantial additional simulation and experimental data have been incorporated into the revised manuscript, strengthening the authors' central claims. The authors have addressed a critical issue in CL imaging—the mechanism of non-local excitation—with a thorough investigation. Therefore, I recommend a Minor Revision pending clarification on the following points.
Q1. The point of our Q1 in the previous review was whether stray electrons are the main source of non-local excitation. The authors calculated the Dy3+ layer's energy absorption caused by electron irradiation on a Si substrate using Monte Carlo simulations. In addition, they demonstrated that energy transfer from adjacent particles (e.g., NaHoF4) is not responsible for non-local excitation by conducting a control experiment using NaGdF4 particles.
However, further clarification is needed to demonstrate that secondary electrons are the excitation mechanism. According to the authors' response, electron irradiation on the Si substrate can effectively excite NaDyF4 particles within ~1 μm, but this effect diminishes significantly beyond 100 μm. Alongside this, the authors show a simulation image in Fig. S9C that even nanoparticles located close to the NaDyF4 layer can be imaged at the 20 nm scale through simulation.
I suggest presenting the image in Fig. S9C with an experimental CL image demonstrating this effect. Experimental validation that stray electrons from the Si substrate can excite nearby nanoparticles, in agreement with the Monte Carlo simulations, would support the authors' claim that stray electrons are the dominant source of non-local excitation.
In the control experiment using non-emissive NaGdF4 particles, it is unclear whether emission from Gd3+ (and possible subsequent energy transfer) could play any energy-transfer role. Could the authors clarify whether such contributions are negligible in this system?

Q2. As mentioned in my previous comment, I still believe describing the relationship between particle diameter and photon detection rate in Figure 4A is inappropriate as a linear (or monotonic increase). Specifically, all the y-intercepts in Figure 4C are negative, significantly deviating from the linear trend observed in Figure 1C or Figure S25C. I would appreciate further clarification from the authors regarding this discrepancy. A proper explanation should be provided if this trend is due to a detection limitation or other experimental constraints.

Reviewer #4

(Remarks to the Author)
This reviewer thanks the authors for their significant work to address the various comments by reviewers. The majority of comments and suggestions have been adequately addressed. Particularly, the figures have significantly improved and are better connected to the narrative text.

However, this reviewer is still troubled by the stated novelty of the work presented here. Other than not analyzing particle aggregates, what is actually developed? It seems that any commercial CL system can produce the same results as long as particles are not aggregated and system conditions are appropriate (e.g. ETD bias voltage, which is system specific due to manufacturers and chamber geometry).

The paragraph at the end of the manuscript beginning with "We achieved single-particle sensitivity by eliminating LNP aggregates from the samples,..." highlights this main concern that persists from the original manuscript. Reducing aggregates does not seem like a development related to CL analysis, but a common sense approach to minimizing signal spread from particle clusters.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors have provided detailed responses to the reviewer's concerns and performed the additional experiments and simulations to address the earlier concerns. These new results reinforce the manuscript's central claims. I believe this work will serve as a valuable resource for researchers in SEM-CL and TEM-CL communities. Accordingly, I recommend the revised manuscript for publication in Nature Communications.

Reviewer #4

(Remarks to the Author)
The reviewer thanks the authors for thoroughly addressing the previous concerns about novelty and contribution to the field at large. The authors clear explanations regarding the ability to definitively isolate the signal from individual LNPs has removed the prior concerns about what was actually developed. The ability to connect the experimental aspects with the modeling effort without artifacts that have been present in previous related publications is highly valuable. The instrumentation issues that were mitigated are both impressive and likely to be of broad interest to the CL community. This will be particularly true for researchers working with LNPs or other highly radiative material systems where material cross-talk is possible. The reviewer also appreciates the modification of the text in lines 258-261 that clarifies the achievements in a more concise manner. Thank you again to the authors for their reponses to previous concerns.

Reviewer #1 (Remarks to the Author):

The manuscript submitted by Rehman et al. presents an investigation into single-particle cathodoluminescence (CL) imaging of small lanthanide nanoparticles, termed "cathodophores." A key challenge identified for multicolor CL imaging is the nonlocal CL excitations caused by secondary electrons. To overcome this issue, the authors utilized a customized spectrometer to selectively isolate characteristic wavelength windows, corresponding to specific dopants. By employing advanced data processing techniques, they were able to identify individual nanoparticle dopants even in densely packed samples. Furthermore, the authors experimentally validated a previously proposed linear relationship between nanoparticle diameter and CL photon detection rate, which is an expected but crucial result.

We thank the reviewer for this apt summary of our work.

Regarding the dependence of CL signal on particle size; we agree that, intuitively, one might expect the CL signal to scale with the diameter of nanoparticles. However, the exact nature of this dependence is not necessarily straightforward. For instance, for optically addressable lanthanide nanoparticles, a linear scaling of brightness with *volume* has been reported[1]. On the other hand, in cathodoluminescence imaging of lanthanide nanoparticles, previous studies have reported CL signal to be independent of particle size[2,3], a result similar to our initial findings (Fig. 1B). This counterintuitive result and discrepancy between optical and electron excitations prompted us to perform Monte Carlo simulations to explore the size dependence of CL signal (SI Fig. 25). The simulations predicted a linear dependence of the nanoparticle brightness on size, a relationship that, to the best of our knowledge, had not been suggested before. Only after understanding the origins and extent of nonlocal excitation (and mitigating it) were we able to demonstrate a monotonic dependence of the CL signal on particle size.

Overall, the manuscript is well written and provides a valuable contribution to the field. The detailed supporting information, especially the images of individual channels, is commendable.

We thank the reviewer for these encouraging comments, highlighting the impact of our findings. Throughout our extensive experience in the field of cathodoluminescence, we were confused by the irreproducibility of single-particle measurements and the inability to achieve multicolor imaging, both by us and other groups. By overcoming nonlocal excitation, we successfully achieved multicolor, single-particle CL imaging, which enabled the results presented in Figs. 3-5. Recognizing that this was the first demonstration of nanoscale, multicolor, single-particle CL imaging, we wanted to do a thorough job of identifying and optimizing the key imaging and sample preparation parameters that impacted cathodoluminescence imaging.

I have a few suggestions that would further improve the manuscript:

1. Consider merging the figures related to nonlocal excitations into a single comprehensive figure (Figure 1). This would enhance clarity and allow for a more cohesive presentation of this important topic.

To comprehensively cover the origin and impact of nonlocal excitation on single-particle imaging, we have now added an illustration to Fig. 1 (Fig. 1E), which shows stray electrons as the hypothesized source of the nonlocal CL signal. Subsequently, Figure 2 now discusses the experiments and simulations that support this hypothesis.

2. While the manuscript introduces multicolor CL imaging, it would be beneficial to clearly highlight the novelty and impact of this approach. Provide precise examples in both the Introduction and Discussion where this technique could be applied. Additionally, outline its advantages over existing ColorEM methods (incl. EDXS, and EELS (in STEM))

To highlight the impact and novelty of our findings and the advantage of CL imaging over existing methods, we have made the following changes to the main text:

6. In the Introduction, we have highlighted the significance of nanoscale optical excitation and far-field imaging offered by CL microscopy, along with its benefits over optical excitation methods including super-resolution imaging (Main text, lines 31-53).

7. In the Introduction, we have highlighted the advantage of CL imaging over other color EM methods, i.e, EELS and EDX, by highlighting its unique ability to probe nanoscale optical properties (Main text, lines 60-64).
8. In the Introduction, we have highlighted the benefits offered by CL imaging in the areas of materials science, quantum sciences, geoscience and bioimaging (Main text, lines 66-76).
9. We have added a new section to the main text to show the impact of our findings in bioimaging. This section shows the imaging of nanoparticles in biological samples, highlighting the applicability of our findings in different systems beyond the Si substrate (Main text, section: Multicolor single-particle CL imaging of LNPs in a biological sample, lines 333-380).
10. In the Conclusion and Outlook, we have added a paragraph on future work that can potentially benefit from our findings, which includes studying intriguing photophysical properties of lanthanide ions, excitons and plasmons in nanomaterials, quantum light sources, and bioimaging applications using cathodoluminescent tags (Main text, lines 413-430).

3. I encourage the authors to explicitly highlight their development that enabled multicolor CL imaging. From my current understanding of the discussion, it appears that minimizing energy deposition in neighboring nanoparticles was essential to achieving this. However, as presented, this outcome seems to stem more from fundamental physical principles rather than a novel experimental or technical advancement. Clarifying this distinction would help underscore the unique contributions of the work.

We agree with the reviewer that the deposition of energy in neighboring nanoparticles due to stray electrons was a fundamental physical principle. To highlight the developments and optimizations that enabled multicolor single-particle CL imaging, we have made the following additions to the main text and the supporting information:

1. We have added new results on the optimization of the chemical composition of lanthanide nanoparticles to achieve their maximal brightness, which was essential for the detection of single nanoparticles, and therefore nanoscale CL imaging (Main text, lines 251-258, and SI, lines 119-129).
2. We have added a discussion on the setup optimizations required to minimize the nonlocal CL signal originating from the scintillator of the SEM's secondary electron detector (Main text, lines 223-233, and SI, lines 776-798).
3. We have included a discussion of our imaging and analysis pipeline in the main text, detailing the detection of nanoparticles emitting only a few hundred photons per second, and the use of long pixel dwell times in CL imaging while addressing sample drift caused by beam-induced charging. Previously, this information was provided only in the supporting information (Main text, lines 259-275, and SI, Section 14, lines 403-483).
4. In the main text, we have highlighted that the electron beam energy and the current was optimized to achieve single-particle CL imaging and have referenced the related sections of the supporting information. Previously these optimizations were not discussed in the main text (Main text, lines 275-276, and SI, Sections 16&17, lines 491-580).
5. We have added new results on the optimizations required to overcome auto-cathodoluminescence from biological cells, required for achieving robust multicolor CL imaging (Main text, lines 337-348).
6. We have also summarized our advancements in sample preparation, instrumentation, and data analysis required to achieve single-particle multicolor imaging in the Conclusion and Outlook section (Main text, lines 400-405).

4. Additionally, the manuscript refers to a "comprehensive framework" at several points, but its precise nature remains unclear to me. Please provide a more detailed explanation of what this framework entails, particularly its role and significance in the context of the study.

We apologize for this confusion. In the main text, we have removed the use of "framework" in favor of more specific language explaining the sample preparation and our imaging and analysis pipeline that enabled robust single-particle CL imaging. We made the following changes to the text:

1. In the abstract, we replace the words *'We present a comprehensive framework to mitigate these nonlocal excitations,'* with *'We mitigate these nonlocal excitations'* (Main text, lines 19-20).
2. In the Introduction, we replace the words *'develop a comprehensive framework'* with *'identify sample preparation conditions'* (Main text, line 103).
3. In Conclusion and Outlook, we replace the words *'By mitigating this nonlocal signal, we established a framework for single-particle CL imaging'*, with *'Identifying the origin of the nonlocal CL signal enabled us to develop the necessary strategies in sample preparation, instrumentation, imaging, spectroscopy, and image analysis to mitigate its impact and consistently achieve multicolor, single-particle imaging'* (Main text, lines 400-402).
4. We have included a discussion of our imaging and analysis pipeline in the main text, detailing the detection of nanoparticles emitting only a few hundred photons per second, and the use of long pixel dwell times in CL imaging while addressing sample drift caused by beam-induced charging. (Main text, lines 259-275, SI, Section 14, lines 403-483).

Reviewer #2 (Remarks to the Author):

In this paper, the authors demonstrate single-particle multicolor CL imaging of lanthanide nanoparticles. Experiments show a scaling law between nanoparticle size and luminescence intensity, and Monte Carlo calculations support the scaling law. These results, while interesting, are predictable for researchers in this field, lack novelty, and are not worthy of publication in Natrue Communications. For publication, we request additional experiments on 1 and 2 below and explanations for 3 and 4.

We are glad that the reviewer found the results presented in the manuscript interesting.

Regarding the scaling of CL signal from nanoparticles as a function of size, intuitively, one might expect the CL signal to scale with the diameter of nanoparticles. However, the exact nature of this dependence is not necessarily straightforward. For instance, for optically addressable lanthanide nanoparticles, a linear scaling of brightness with *volume* has been reported[1]. On the other hand, in cathodoluminescence imaging of lanthanide nanoparticles, previous studies have reported CL signal to be independent of particle size[2,3], a result similar to our initial findings (Fig. 1B). This counterintuitive result and discrepancy between optical and electron excitations prompted us to perform Monte Carlo simulations to explore the size dependence of CL signal (SI Fig. 25). The simulations predicted a linear dependence of the nanoparticle brightness on size, a relationship that, to the best of our knowledge, had not been suggested before. Only after understanding the origins and extent of nonlocal excitation and mitigating it were we able to achieve a monotonic dependence of the CL signal on particle size.

Overcoming nonlocal excitation was the crucial advancement that enabled single-particle imaging. This advancement allowed us to develop optimized sample preparation, imaging, and analysis pipelines for nanoscale CL imaging. These developments were crucial to achieving the multicolor imaging results presented in Fig. 3. In addition, our findings allowed us to leverage the full nanoscale imaging potential of CL imaging, as demonstrated by our ability to characterize single nanoparticles, which had not been possible before. Lastly, we have now added an additional section in the main text to demonstrate the wider applicability of our findings across diverse systems by performing multicolor, single-particle CL imaging in biological samples (Fig. 5). We believe that together these findings present a significant advancement in the field.

We thank the reviewer for their fair and important comments, which we address below.

1. When applying this imaging technology to biological and materials science, there will be few situations where only lanthanide nanoparticles will be observed. There is a concern that sufficient spatial resolution and luminescence intensity may not be accomplished due to the influence of surrounding materials. The authors need to show single-particle multicolor CL imaging data for biological or materials science applications and discuss the luminescence properties of single lanthanide nanoparticles, including their spatial resolution, luminescence intensity, and CL background.

We agree with the reviewer that when imaging lanthanide nanoparticles in biological or materials science applications, background cathodoluminescence and its impact on nonlocal CL signal could present a

challenge. To demonstrate the applicability of our findings in biological systems, we have added a new section to the main text along with an accompanying figure (Fig. 5).

In this section, we discuss the impact of auto-cathodoluminescence from cells on the CL imaging of nanoparticles, and present a sample preparation technique to mitigate this background signal. We then demonstrate multicolor single-particle imaging on the surface of a mammalian cell. We also compare the brightness of nanoparticles in the biological sample and on a Si substrate (Main text section: Multicolor single-particle CL imaging of LNPs in a biological sample, lines 333-380).

We also compare the size of the LNPs on the Si substrate in biological samples, and demonstrate that, other than the impact of sputter coating which reduces charging during imaging, no loss of spatial resolution is observed (Main text, lines 365-368, and SI, lines 744-762).

2. Regarding the above question, fluorescence imaging is also necessary for the areas observed by CL imaging. This is correlative imaging by CL and fluorescence.

We wish to emphasize that our CL imaging method is not correlative. For all samples in our manuscript, including the newly added biological sample, all CL imaging is done using an SEM without the need for accompanying fluorescence images. We apologize if this comment is not fully answered yet and are happy to address it further if more context is provided.

3. The authors use the term secondary electrons in the paper, how did they prove this? Is it possible to ignore the possibility that e.g. reflected electrons or characteristic X-rays could have excited the surrounding lanthanide nanoparticles?

We agree with the reviewer that any type of electrons scattered from the excited nanoparticle upon interaction with the electron beam could cause nonlocal excitation. We apologize for not making this distinction in our earlier draft. We now use language that encompasses electrons beyond strictly secondary electrons (i.e., “stray electrons”) when discussing the cause of the nonlocal CL signal.

Furthermore, to demonstrate that characteristic X-rays are not likely to cause this nonlocal CL signal, we performed additional experiments. Briefly, by using a material that is transparent to the characteristic X-rays of elements in the nanoparticles, but that blocks electrons (i.e., a 200-nm-thick Si_3N_4 window), we show the mitigation of the nonlocal CL signal. We have now presented a discussion on this point in the main text and the supporting information (Main text, lines 220-222 and SI, lines 223-255).

4. When using NaYF4:Dy, increasing the concentration of Dy causes concentration quenching, which lowers luminescence efficiency. Please explain the reason for using NaLnF4.(Ln = Lanthanide)

We agree with the reviewer that concentration quenching can affect the brightness of nanoparticles as the concentration of dopants increases. However, in our CL imaging studies, we observed that the increase in CL signal due to higher dopant concentrations and the effect of concentration quenching counterbalance each other, resulting in no significant change in CL signal from the nanoparticles beyond 20% dopant concentration. We have added single-particle CL measurements at different concentrations of dopants in the supporting information along with a brief discussion in the main text (Main text, lines 251-258 and SI, lines 119-129).

Reviewer #3 (Remarks to the Author)

The manuscript "Multicolor Cathodoluminescence from Single Lanthanide Nanoparticles" by S. A. Rehman addresses a critical limitation to achieving nanometer-scale multicolor cathodoluminescence (CL) imaging. It demonstrates that the resolution of the multicolor CL imaging can be improved to a few tens nanometer scale. The authors experimentally demonstrate a potential nonlocal excitation pathway by designing a sample with a concentrated cluster of particles on one side and sparsely distributed single particles on the other. Using carefully prepared samples that do not contain aggregated particles, the authors also demonstrate highly spatially-resolved multicolor imaging with a resolution of ~12 nm. Moreover, the CL-intensity curve as a function of particle diameter presents interesting results, showing the quantitative nature of CL from nanoparticles. The information in the manuscript will be of interest to the SEM and TEM-CL communities.

The author effectively demonstrates potential misinterpretations that can arise during CL multi-color imaging through well-structured experiments, and the size-dependent data is quite intriguing. However, sub-20 nm scale CL multicolor imaging using SEM and TEM has already been reported in other studies (Swearer, D. F., et. al., ACS Photonics 2021, 8, 1539-1547; Mauser, K. W., et. al., ACS Nano 2021, 15, 11385-11395).

We thank the reviewer for their summary and for highlighting the impact of our work.

Regarding other studies reporting sub-20 nm scale CL imaging, we would like to emphasize that, despite previous efforts, including the studies mentioned by the reviewer, multicolor CL imaging has not yet been demonstrated at nanoscale resolution (including sub-20 nm scale). As we demonstrate in this manuscript, this limitation arises due to the nonlocal CL signal caused by stray electrons.

The study by Swearer, D. F. et al., *ACS Photonics* 2021, mentioned by the reviewer, could not distinguish between spectrally distinct nanoparticles within a field of view (Fig. 4E&F, reproduced here as Fig. 1A).

[Figure Redacted]

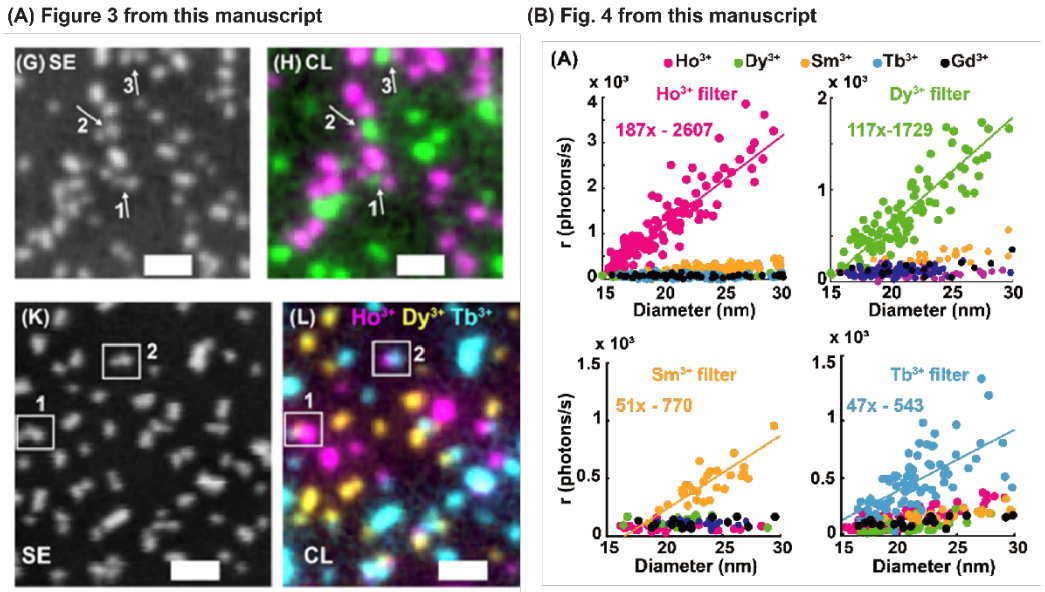

Figure 3: CL analysis presented in our paper. (A) Multicolor single-particle CL imaging (Scale bars: 100 nm). (B) Dependence of the Cl signal on the size of the lanthanide nanoparticles.

Importantly, the authors explicitly mention their lack of spectral resolution in CL imaging (text from the paper: *'Figure 4F, however, revealed no distinguishable spatial resolution for the filtered panchromatic CL images').* This lack of spatial resolution in multicolor CL imaging also brings into question the single-particle spectral measurements presented in the paper (Fig. 3), as these measurements could also be impacted by the nonlocal excitation. In addition, the study did not find any relationship between the size of the nanoparticles and their brightness (Fig. S6A, reproduced here as Fig. 1B). This independence of CL brightness and nanoparticle size matches our initial findings, where we attributed this behavior to the nonlocal excitation of clusters due to stray electrons originating from the excited particle.

Similarly, in the study by Mauser, K. W. et al., *ACS Nano* 2021, temperature changes along GaN nanowires were measured using the wavelength shift of CL emission as an indicator. However, the resolution was constrained by the size of the interaction volume, which at 5 keV (as used in the study) was approximately 200 nm (Fig. S2A). As a result, temperature variations over scales of hundreds of nanometers were presented (Fig. 3A, reproduced here as Fig. 2).

Here, we wish to add that earlier efforts to achieve multicolor CL imaging have been limited to large nanoparticle aggregates, hundreds of nanometers to micrometers in size[4,5]. In contrast, we demonstrate multicolor single-particle CL imaging with 12 nm to ~30 nm lanthanide nanoparticles in the same field of view (Fig. 3A, reproduced here as Fig. 3A). Furthermore, we also show a monotonic increase in the CL signal as a function of the size of the particle (Fig. 4A, reproduced here as Fig. 3B).

We believe that this manuscript is the first demonstration of nanometer scale, multicolor CL imaging. We discuss the requirements to demonstrate multicolor CL imaging in the main text as:

'Nonlocal excitation could result in the apparent emission of single LNPs across multiple spectral channels. Therefore, demonstrating multicolor imaging of spectrally distinct LNPs in the same field of view was crucial to confirm single-particle detection capability. Conversely, single-color imaging alone was insufficient to establish single-particle detection sensitivity, even when the CL signal from LNPs spatially coincided with their image in the SE channel.' (Main text, lines 236-241)

I agree that the aggregation of LNPs is the main source of nonlocal excitation as presented in Figure 1. However, the evidence supporting the conclusion that secondary electrons are the cause of nonlocal excitation appears to be insufficient. There seems to be a great discrepancy in the length scale between the Monte Carlo simulation results and the distance-dependent intensity results shown in Figure 2. Therefore, the interpretation of the non-local excitation and the size-dependent results does not appear to be convincing, and thus the analysis does not seem to convey novel scientific insights.

We apologize for the confusion caused by the distance-dependent measurements shown in Figure 2 and the Monte Carlo simulations of two adjacent nanoparticles presented in Figure 3. The simulations were conducted at short distances and did not correspond to the conditions depicted in Figure 2, as they excluded LNP aggregates. To address this confusion, we have included new Monte Carlo simulations that demonstrate the influence of stray electrons on the origin of nonlocal CL signals over longer distances in a setup similar to the experiment in Figure 2. These simulations validate and support the distance-dependent intensity results shown in Figure 2.

Considering the above two aspects, unfortunately, this research does not seem to present a significant scientific advancement to meet the high standards required for publication in Nature Communications. I would recommend the authors to submit the work elsewhere once the following concerns and suggestions are properly addressed:

We believe we have addressed the two primary points raised by the reviewer and are happy to provide additional information if needed.

We would like to take this opportunity to thank the reviewer for their encouraging remarks on the importance of our work in achieving nanoscale multicolor cathodoluminescence imaging and its significance in identifying the misinterpretation of multicolor CL imaging data. We are pleased to hear that the reviewer found our experiments in this regard well-structured. In addition, we are grateful for the reviewer's acknowledgment of the importance of cathodoluminescence signals scaling as a function of nanoparticle size, and for recognizing the broader significance of our work to the electron microscopy community.

Having addressed the reviewer's two main concerns, along with the major and minor comments below, and given the significance of our contributions, we respectfully request the reviewer to reconsider their decision regarding the publication of our manuscript in *Nature Communications*.

Major comments.

Q1-1. In Figure 3A, the authors show that less than 2% of the energy is deposited into an adjacent LNP that is 20 nm away from the particle excited by the electron beam. The energy absorption decays exponentially (or according to a quadratic function) with distance and becomes almost zero when the particles are separated by approximately 200 nm. This is three orders of magnitude shorter than the distance at which the Monte Carlo simulation shows almost zero probability of energy absorption. This points out that the secondary electrons from a single particle are unlikely to excite aggregated particles located 200 μm away, which

We apologize for the confusion caused by the distance-dependent measurements shown in Figure 2 and the Monte Carlo simulations of two adjacent nanoparticles presented in Figure 3. The simulations were conducted at short distances and did not correspond to the conditions depicted in Figure 2, as they excluded LNP aggregates. To address this confusion, we have included new Monte Carlo simulations that demonstrate the influence of stray electrons on the origin of nonlocal CL signals over longer distances in a setup similar to the experiment in Figure 2. These simulations validate and support the distance-dependent intensity results shown in Figure 2.

Also, although the LNP contains heavier elements than the Si substrate, the secondary-electron generation event and its absorption by aggregated particles would be more significant when the electron beam is focused on the Si substrate, which is near the aggregated particles, compared to a single particle 200 μm away from the aggregated particles. This suggests that secondary electrons may not be the primary source of nonlocal electron excitation again.
In this regard, the authors may consider distance-dependent Monte Carlo simulation results to check the number of secondary electrons generated on the Si substrate and absorbed by a single particle, similar to what is shown in Figure 3A?

We agree that the nonlocal CL signal would be higher when the electron beam is focused on the Si substrate near the aggregated particles compared to the nonlocal CL signal due to a single nanoparticle 200 μm away from the aggregated particles. However, at both distances, the nanoparticles would exhibit a higher nonlocal signal relative to the surrounding (local) Si substrate, creating the illusion that the nanoparticle is 'emitting' above the background CL from the local Si substrate. To further clarify this point, we have performed the simulations suggested by the reviewer, which are now included in the supporting information along with a discussion in the main text (Main text, lines 171-176 and SI, lines 202-221).

Q1-2. If secondary electrons are not the source, energy transfer (Förster- or Dexter-type?) could be the origin of the nonlocal excitation. This hypothesis could be tested using a system similar to that in Figure 2, but with non-emissive particles that generate a similar number of secondary electrons as $NaHoF_4$, instead of $NaHoF_4$ itself. If the $NaDyF_4$ spectrum is no longer observed, it would suggest that the origin of the nonlocal excitation observed in single particles originated from energy transfer from the single particle to the aggregated particles.

Following the reviewer's recommendation, we conducted experiments using sparse non-emitting nanoparticles ($NaGdF_4$) and clustered emitting nanoparticles. Gd^{3+}, Ho^{3+} and Dy^{3+} have very similar atomic numbers, and we expect them to generate a similar number of stray electrons. When imaging the sparse non-emitting nanoparticles, we observed a signal in the channel corresponding to the emission spectrum of the clustered particles, which supports the hypothesis that the nonlocal CL signal is primarily caused by stray electrons (SI, lines 257-267).

Furthermore, to rule out photonic emission (such as characteristic X-rays) as the likely cause of the nonlocal CL signal, we performed additional experiments. Briefly, by using a material that is transparent to the characteristic X-rays of elements in the nanoparticles, but that blocks electrons (i.e., a 200-nm-thick Si_3N_4 window), we show the mitigation of the nonlocal CL signal. This again implicates stray electrons as the cause of the nonlocal CL signal (Main text, lines 220-222 and SI, lines 223-255).

Q2-1. The experimental observation of CL-intensity dependence on particle size, along with its verification through simulation results, is a fascinating finding that could significantly enhance the understanding of the quantitative nature of CL from nanoparticles in both the SEM CL and TEM CL communities. However, I'm unsure if we can confidently describe the relationship between radius and intensity in Figure 4A as linear. For example, in the first panel of Figure 4A, the intensity of the 20-nm particle is around 1, while for the 30-nm particle, it is 3. Could it be cubic (volume) dependence?

We thank the reviewer for appreciating the significance of the scaling of CL intensity as a function of particle size, as supported by our simulations, and for recognizing its importance to the broader electron microscopy community.

We believe that the observed variability in the brightness of nanoparticles of a given size was likely due to differences in their axial dimensions, as their height (axial diameter) was estimated from their lateral dimensions. In addition, electrobleaching may have contributed to further variability in particle brightness. We have included a discussion on these points in the main text (Main text, lines 277-286).

Furthermore, we have also compared linear and nonlinear (cubic and quadratic) fits to the brightness-size data. Our analysis showed no improvement in fit quality when using nonlinear models. While we chose a linear model to describe the data due to its simplicity and its agreement with the prediction from the Monte Carlo simulations, we have not ruled out the possibility of a nonlinear dependence. We have added a discussion on this consideration in the main text, and included different fits in the supporting information (Main text, lines 312-317 and SI, lines 660-687).

Q2-2. According to page 24 of the supporting information, this linearity breaks down around 15 nm when the electron beam energy is reduced. Have you experimentally observed this breakdown in linearity using an electron beam with an energy lower than 0.5 keV?

Unfortunately, we could not achieve such low electron beam energies in our SEM-CL system. This limitation was due to the requirement of long working distances in our system to accommodate the positioning of the parabolic mirror between the sample and the electron gun. When imaging with low beam energies at long working distances, especially through the aperture of the parabolic mirror, we observed a significant loss of resolution. We have now highlighted this limitation in the main text and the supporting information (Main text, lines 317-324 and SI, lines 534-552).

Q3. In some lanthanide-doped particles, the CL intensity may not increase linearly with the electron-dose rate, as excessive excitons and free carriers generated under high dose rates can alter the population distribution of emitting excited states. Were the measurements or simulations in the manuscript conducted with dose-rate dependence taken into account, or were they performed within the regime where intensity increases linearly with the electron-dose rate?

We investigated the impact of the beam current on the brightness of nanoparticles. We did not observe a change in CL emission as a function of beam current, which could be due to increased electrobleaching at higher currents counteracting any increase in the emission rate caused by the higher current. This is described in the supporting information, and we have now included references to the optimizations of beam energy and current in the main text (Main text, lines 275-276 and SI, lines 491-513).

Minor comment.
Q1. In the first section, 'Nonlocal electron excitation can prohibit single-particle multicolor CL imaging', it is suggested that nonlocal electron excitation dominates multicolor CL imaging, hindering both quantitative and qualitative analysis of LNP emission. And, in the following section, minimizing the LNP aggregation mitigates nonlocal electron excitation. As far as I understand, the only difference between the experimental conditions in Figures 1 and 3 is the distribution of LNPs on the Si substrate, which determines the presence of nonlocal electron excitation. However, since only a single particle is shown in Figure 1, it is difficult to imagine that aggregated LNPs are nearby (within 200 μm). Could you provide a lower-magnification image of the sample in Figure 1?

We thank the reviewer for this suggestion. We have now added an illustration in Figure 1E to show the origin of nonlocal CL signal due to the excitation of clusters outside the imaged region of the sample. We have also

provided example low magnification images showing nanoparticle aggregates, along with high magnification images to visualize single nanoparticles, in the supporting information (SI, lines 270-280).

Q2. Could you please define "CL brightness"? Is it different from the number of photons?

We apologize for not clearly defining the term "CL brightness" earlier. By CL brightness, we refer to the number of photons detected per second from a nanoparticle. We have now defined this term in the main text when it was first mentioned and included a reference to the analysis performed to calculate it from the CL images, as described in SI Section 14 (Main text, lines 250-251).

Reviewer #4 (Remarks to the Author):

What is the difference between "multi-color CL" and CL spectrum imaging? It seems unnecessary to rename an established technique.

We choose the term “multicolor CL” over “CL spectrum imaging” because our CL imaging method uses distinct emission bands to distinguish color, and does not involve collecting CL emission spectra. Also, several other studies have used the term “multicolor cathodoluminescence” previously (see below). For these two reasons, if at all possible, our preference would be to keep the original nomenclature.

- Niioka, H., Furukawa, T., Ichimiya, Ashida, M., Araki, T., & Hashimoto, M. (2011). Multicolor Cathodoluminescence Microscopy for Biological Imaging with Nanophosphors. Applied Physics Express, 4(11), 112402.
- Taichi Furukawa, Shoichiro Fukushima, Hirohiko Niioka, Naoki Yamamoto, Jun Miyake, Tsutomu Araki, & Mamoru Hashimoto. (2015). Rare-earth-doped nanophosphors for multicolor cathodoluminescence nanobioimaging using scanning transmission electron microscopy. Journal of Biomedical Optics, 20(5), 056007.
- Bellocchi, G., Fabbri, F., Miritello, M., Iacona, F., & Franzò, G. (2015). Multicolor Depth-Resolved Cathodoluminescence from Eu-Doped SiOC Thin Films. ACS Applied Materials & Interfaces, 7(33), 18201–18205.

It is not clear what value Figure 1b and 1c add to the discussion. Also, they are plotted on different y-axis scales. If they are necessary please specify why in the text and plot both on either the exponential scale or linear scale, not one on either.

We apologize for not adequately emphasizing the significance of Figures 1B and 1C. To address this, we have now plotted Figures 1B and 1C with a linear y-axis and added a discussion in the main text to highlight their importance in the context of nonlocal CL signal.

Briefly, Figure 1B presents the results of the experiments to characterize CL emission from single nanoparticles, in samples containing both single nanoparticles and aggregates. In these experiments we observed that the brightness of particles was independent of their size. While this result was consistent with previous studies, it did not match our Monte Carlo simulations, which suggested a linear increase in CL brightness as a function of particle size. However, when we overcame nonlocal excitation, the brightness-size measurements matched the predictions of the Monte Carlo simulations, as shown in Fig. 4A.

Hence, Figure 1B and 1C highlight the discrepancy between experimental observations and simulations, which we attributed to nonlocal excitation. We later discuss the origin and extent of this nonlocal excitation, demonstrate how it was mitigated, and show that in the absence of nonlocal excitation, the experimental results align well with the simulations (Main text, lines 121-131 and 277-281).

It is clear from the spectra in Figure 1a that the Dy sample has an emission peak that overlaps with what is defined as the Ho channel. Without showing the spectra (not normalized) for the plots in 1d I am left wondering if the Ho map is just representing part of the Dy emission around 650nm.

We agree with the reviewer that a minor peak of Dy^{3+} ions overlaps with the main emission peak of Ho^{3+} ions. However, we believe that the signal in the Ho^{3+} channel in Figure 1A was not caused by this overlap. This is supported by Figure 1A, where no signal is observed in the Ho^{3+} channel for a sample containing only $NaDyF_4$

nanoparticles. Furthermore, in brightness-size measurements for single nanoparticles presented in Figure 4A, the signal from $NaDyF_4$ nanoparticle closely matched the non-emitting control nanoparticles in the Ho^{3+} channel (green data points in Ho^{3+} filter).

Figure 2d does not seem to agree with the discussion in the text. The text says that a thick layer of NaDyF4 is separate from a sparse coverage of NaHoF4 particles. 2d shows a mix of particles, correct this to match the text discussion or state why it is different.

We apologize for the confusion caused by the original Figure 2, which showed an illustration (depicting aggregates containing LNPs of different colors) next to the experimental data (containing aggregates of a single color). To address this, we have added a Monte Carlo simulation (new Fig. 2D) corresponding to the experimental data discussed in Figures 2A–C. These simulations validate and support the experimental findings shown in Figure 2.

The previous illustration, i.e., previously Figure 2D of the original manuscript, has been moved to Figure 1E to show the hypothesized origin of nonlocal CL signal.

The sentence "Interestingly, since the LNPs were composed of heavier elements than the Si substrate, they produced more secondary electrons." needs revision if it is necessary. It is not "interesting" that higher Z elements emit more SEs, this is a fact included in any introductory discussion of SE generation.

We agree with the reviewer that the increased generation of secondary electrons from heavier elements is not a new observation. However, the resulting spatial correlation between the increase in the nonlocal CL signal and the spatial location of the nanoparticles makes it tempting to erroneously categorize this CL signal as originating from the nanoparticle, and challenging to distinguish it from the true CL signal from the nanoparticle. We have revised the text to clarify this point:

'Interestingly, although the nonlocal CL signal appeared to originate from the spatial location of the imaged LNP, this was an artifact. This is because the heavy metal composition of the LNPs caused them to generate more stray electrons than the surrounding Si substrate, resulting in the localized amplification of the nonlocal signal at the LNP compared to the Si substrate.' (Main text, lines 164-168)

This statment " The simulations showed that for two neighboring LNPs, if one was excited with the electron beam, <2% of the energy was deposited into the neighboring LNP, even if the two LNPs were in direct contact. Such minimal crosstalk indicated that multicolor CL imaging with nano-crystals should be possible in samples containing only individual non-aggregated LNPs" is quite concerning.

How can the authors argue that the Ho signal from the Dy particles is an artifact if the above statement is accurate? Figure 1d shows one particle in the field of view, not even "touching" another particle. Why would there be an issue with the emission from such a particle? clearly there is not as in Figure 3 the authors claim all is well and there are no artifacts present. The only "mitigation" that was presented seems to be a simulation and then preparation of a more sparse distribution of particles. Though even the latter part of that statement seems wrong as in Figure 3 the particles are close or touching but with no issues with artifacts.

We apologize for the confusion caused by the presentation of our experiments and simulations on single-particle imaging in the presence and absence of nanoparticle aggregates. To improve clarity, we have restructured the figures and revised the text to better distinguish between these conditions.

Briefly, in Fig. 1, we set up the problem by demonstrating that spectrally distinct signals could be obtained from samples containing a *single type* of lanthanide nanoparticles (i.e., nanoparticles doped with either Ho^{3+} or Dy^{3+} ions). However, in samples containing a mixture of different ions, multicolor imaging was hindered due to nonlocal excitation of multicolored aggregates of nanoparticles that were outside the scanned region but within the field of view of the mirror. This concept is illustrated in Fig. 1E. We implicated stray electrons as the cause of this nonlocal signal. In addition, we have included SI Fig. 12 to provide examples where nanoparticle aggregates were absent in the scanned region at the magnifications required to image single nanoparticles, but became visible in lower magnification images.

Fig. 2 discusses an experiment to test the hypothesis that nonlocal signal was caused by stray electrons. This experiment demonstrated that stray electrons excited nanoparticle aggregates located hundreds of micrometers away. We also present new Monte Carlo simulations of the experiment presented in Fig. 2, which matched the experimental results.

Then, in Fig. 3 we show that in the absence of nanoparticle aggregates, nonlocal excitation did not pose a problem and multicolor imaging was possible with spectrally distinct nanoparticles in the same field of view. Importantly, we demonstrated that sparse nanoparticle samples were not a prerequisite for multicolor single-particle CL imaging, as long as nanoparticle aggregates are absent. We also discuss crosstalk between neighboring nanoparticles in dense, but non-aggregated, samples in SI Figs. 40&41.

In Figs. 4&5, we demonstrate the utility of our single-particle sensitivity to characterize the emission of individual lanthanide nanoparticles (again, in the absence of aggregates) and the broader applicability of our method by imaging single nanoparticles in biological samples.

We hope that these revisions clarify the data presentation and address the confusion caused by the original organization.

In the conclusion section there is the statement "The key advancement that enabled localized CL excitation was overcoming nonlocal CL signal caused by stray secondary electrons. These stray electrons could excite nano-crystals hundreds of micrometers away from the point of beam-sample interaction. " From the points described above I find this advancement to be misleading. The overcoming of nonlocal signal seems to be by making particles spaced farther apart (a logical and elementary solution) but then the authors show in Figure 3 that this doesn't matter... Yes, if you directly excite particle aggregates you can get signal from multiple particles as evidenced by the monte carlo simulations but why single particle analysis is an issue is not clearly presented in this manuscript.

We apologize for the confusion caused by the presentation of our experiments and simulations on single-particle imaging in the presence and absence of nanoparticle aggregates. We agree that directly exciting nanoparticle aggregates will result in CL signals from multiple nanoparticles within the aggregate. However, stray electrons originating from exciting a *single* nanoparticle can also excite nanoparticle aggregates, even if they are located outside the scanned region, as illustrated in Fig. 1E. If these aggregates are within the field of view of the mirror, their CL signal is still collected. Importantly, despite the nonlocal nature of this CL signal, it appears to be spatially localized to the position of the excited nanoparticle in the CL image. This makes it challenging to distinguish from the true CL signal of the particle itself. This nonlocal excitation significantly limits both multicolor imaging and accurate single-particle brightness characterization.

We hope our new organization of the figures and revisions to text, as discussed above, will clarify this point.

Reviewer #5 (Remarks to the Author):

In the manuscript titled Multicolor Cathodoluminescence from Single Lanthanide Nanoparticles, Sohaib Abdul Rehman et al. performed the cathodoluminescence in lanthanide nanoparticles and present intriguing findings.

The results address the issue of nonlocal excitation in multicolor cathodoluminescence (CL) imaging, facilitating single-particle multicolor CL imaging. This study enhances both the resolution and accuracy of imaging while offering new insights into nano-research. However, minor revisions are needed before this manuscript can be accepted for publication in Nature Communications.

We thank the reviewer for their encouraging remarks.

1. **The abstract could benefit from being more concise.**

We have now edited the abstract for conciseness and clarity.

2. **The authors should include more information on recent advancements in optical excitation (photoluminescence, PL) compared to electron beam excitation (cathodoluminescence, CL) in the introduction. This will highlight the significance of the article's findings.**

We thank the reviewer for this suggestion. In the introduction, we have now included a discussion on the significance of probing nanoscale optical properties of samples in bioimaging and materials science. We highlight the importance of photoluminescence in studying these properties, as well as the inherent resolution limit imposed by the diffraction of light. We also discuss recent advancements in super-resolution microscopy to overcome the diffraction limit in fluorescence imaging. However, we also address the limitations of these techniques, particularly their reliance on specifically designed fluorescent probes, which restrict their applications. This discussion leads to the advantages offered by cathodoluminescence in achieving nanometer-scale resolution (Main text, lines 31-53).

3. The data presented in Figure 3 are adequate to illustrate the impact of secondary electrons (SE); if possible, please include more detailed information.

In the main text, we have further clarified the point that the nonlocal CL signal was primarily caused by aggregates of nanoparticles and that multicolor imaging became feasible even in dense nanoparticle samples upon removing these aggregates (Main text, lines 212-220).
We have also added new experiments that support the hypothesis that the nonlocal excitation was caused by stray electrons rather than photonic emission originating from the excited nanoparticle (Main text, lines 220-222 and SI, lines 223-255).

4. Despite the low accelerating voltage of 3 kV used in the manuscript, some damage to the nanoparticles still occurs, affecting imaging accuracy. Please include the results of reproducibility experiments to address this issue.

We had a discussion on the stability of the nanoparticles under the electron beam in the SI. We apologize for not referring to it in the main text. We have now updated the main text to include a discussion on the electrobleaching of nanoparticles and have referenced the related SI Figs. 32&33 (Main text, lines 284-286 and SI, lines 706-721).
We have also included a discussion on the reproducibility of the CL signal from nanoparticles across different syntheses in the supporting information (SI, lines 816-822).

5. The manuscript would benefit from proofreading by a native English speaker or a professional language editing service.

Thank you, we have further edited the manuscript for clarity.

Reviewer #6 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you.

References

1. Ren, P. *et al.* Quantifying the influence of inert shell coating on luminescence brightness of lanthanide upconversion nanoparticles. *ACS Photonics* **9**, 758–764 (2022).
2. Swearer, D. F. *et al.* Single Particle Cathodoluminescence Spectroscopy with Sub-20 nm, Electron-Stable Phosphors. *ACS Photonics* **8**, 1539–1547 (2021).
3. Prigozhin, M. B. *et al.* Bright sub-20-nm cathodoluminescent nanoprobes for electron microscopy. *Nat. Nanotechnol.* **14**, 420–425 (2019).
4. Niioka, H. *et al.* Multicolor Cathodoluminescence Microscopy for Biological Imaging with Nanophosphors. *Appl. Phys. Express* **4**, 112402 (2011).
5. Garming, M. W. H. *et al.* Nanoparticle discrimination based on wavelength and lifetime-multiplexed cathodoluminescence microscopy. *Nanoscale* **9**, 12727–12734 (2017).

Reviewer #2 (Remarks to the Author):

The authors have addressed the main concerns raised in the previous review round. In particular, they have conducted a sufficient number of additional experiments and provided new figures, which help to clarify the points in question.

We thank the reviewer for their important comments in the previous review round. They have helped us improve our work.

The current version of the manuscript represents a meaningful improvement over the previous submission. I believe the manuscript is now acceptable for publication.

We thank the reviewer for their encouraging remarks.

Reviewer #3 (Remarks to the Author):

I appreciate the authors' detailed responses and the additional simulations to address this question. Substantial additional simulation and experimental data have been incorporated into the revised manuscript, strengthening the authors' central claims. The authors have addressed a critical issue in CL imaging—the mechanism of non-local excitation—with a thorough investigation. Therefore, I recommend a Minor Revision pending clarification on the following points.

We thank the reviewer for their encouraging remarks on the significance of our work in investigating the mechanism of nonlocal excitation in CL imaging. We have performed additional experiments and simulations to address the reviewers' points.

Q1. The point of our Q1 in the previous review was whether stray electrons are the main source of non-local excitation. The authors calculated the Dy3+ layer's energy absorption caused by electron irradiation on a Si substrate using Monte Carlo simulations. In addition, they demonstrated that energy transfer from adjacent particles (e.g., NaHoF4) is not responsible for non-local excitation by conducting a control experiment using NaGdF4 particles.
However, further clarification is needed to demonstrate that secondary electrons are the excitation mechanism. According to the authors' response, electron irradiation on the Si substrate can effectively excite NaDyF4 particles within ~1 μm, but this effect diminishes significantly beyond 100 μm. Alongside this, the authors show a simulation image in Fig. S9C that even nanoparticles located close to the NaDyF4 layer can be imaged at the 20 nm scale through simulation.
I suggest presenting the image in Fig. S9C with an experimental CL image demonstrating this effect. Experimental validation that stray electrons from the Si substrate can excite

nearby nanoparticles, in agreement with the Monte Carlo simulations, would support the authors' claim that stray electrons are the dominant source of non-local excitation.

We thank the reviewer for this comment. To further clarify that stray electrons were indeed responsible for nonlocal excitation and that they also occurred when imaging the substrate, we have now added experimental validation of the simulation results presented in SI Fig. 9. Specifically, in SI Fig. 9, we have included a plot comparing CL signals from $NaHoF_4$ LNPs and Si when positioned at different distances from a thick $NaDyF_4$ layer, along with images showing LNPs and their surrounding Si substrate at these distances. Cross-sectional profiles of these images are also included in the figure. In addition, in Fig. 2 of the main text, which also shows images of $NaHoF_4$ LNPs at different distances from the $NaDyF_4$ layer, we have now included the scaling of the images' pixel values in the caption.

Furthermore, we have now modified Fig. 2 of the main text with more experiments investigating the nonlocal CL signal. We include experiments for Si_3N_4 and 80:20 Pt:Pd substrate, and for different nanoparticles, both lanthanide and non-lanthanide. In all these experiments, we show that the nonlocal signal 'from' the excited particle depends on the difference in the atomic number of the particles and the substrate. For instance, LNPs on Si (atomic number 14) display nonlocal CL signal with much larger contrast than LNPs on 80:20 Pt:Pd (atomic numbers 78 and 46) do. Moreover, we observe a shadowing effect: the CL signal from the substrate decreases behind the LNP, because the LNP blocked stray electrons originating from the substrate, thereby preventing them from reaching the thick layer. This effect was more pronounced on the 80:20 Pt:Pd substrate due to its higher atomic number. This shadowing effect was also predicted by Monte Carlo simulations, which model only electron trajectories and exclude any radiative or non-radiative energy transfer. (Fig. 2 and SI Figs. 9–14)

Together, these additional results support our conclusion that nonlocal excitation occurs in both the LNPs and the substrate. Due to the higher atomic number and topography of the particles, they generate more stray electrons, leading to the appearance that the nonlocal signal is spatially localized at the excited LNPs.

In the control experiment using non-emissive NaGdF4 particles, it is unclear whether emission from Gd3+ (and possible subsequent energy transfer) could play any energy-transfer role. Could the authors clarify whether such contributions are negligible in this system?

We thank the reviewer for this comment. To clarify whether radiative or non-radiative energy transfer from excited LNPs (either emitting or non-emitting) to the thick layer of LNPs could be responsible for nonlocal CL in the absence of stray electrons, we performed two sets of experiments: (i) samples where stray electrons were blocked but X-rays and other photonic excitations could still occur, and (ii) using different types of substrates and excited LNPs to show that nonlocal excitation is not an intrinsic property of the excited particle or the substrate, i.e., the electronic properties do not matter.

For the first case, we used a medium that is transparent to photonic emission (X-rays and optical) but blocks or attenuates stray electrons. This was achieved using a 200-nm Si_3N_4 window, with sparse LNPs on the top side and a dense $NaHoF_4$ layer on the bottom side. We performed the experiment under two conditions: exciting either sparse $NaDyF_4$ or control $NaGdF_4$ LNPs. In both cases, we observed attenuation of the signal in the $NaHoF_4$ channel at the location of the excited LNPs, indicating that radiative energy transfer is not responsible for the nonlocal signal. (Fig. 2 and SI Figs. 10&13)

We confirmed that nonlocal CL excitation could still occur with Si_3N_4 as the substrate in a configuration similar to the experiment performed on Si. We placed dense $NaHoF_4$ on approximately half of the Si_3N_4 surface area, and sparse $NaGdF_4$ on the other half. In this configuration, we did observe nonlocal excitation. (Fig. 2 and SI Fig. 13)

We also repeated this experiment on an 80:20 Pt:Pd substrate, where we also observed nonlocal signals extending over hundreds of micrometers. Moreover, we saw a reduction in the CL signal from the substrate behind the LNP, which was corroborated by Monte Carlo electron trajectory simulations. Since simulations only model electron trajectories and not any radiative or non-radiative effects from LNPs, our hypothesis is supported. (Fig. 2 and SI Fig. 14)

In addition, to rule out any lanthanide-specific effects from Gd^{3+} ions, we performed similar experiments using non-lanthanide nanoparticles, $NaYF_4$ and gold (Au), and observed nonlocal signals in both cases. (Fig. 2 and SI Fig. 12)

For all the experiments involving different substrates and different excited sparse particles, the nonlocal signal was dependent on the difference in the atomic number between the excited particle and the substrate. Together, these results support the conclusion that nonlocal signal is not an intrinsic property of the excited particle or the substrate but rather is independent of the chemical nature of the nanoparticle, and depends only on its atomic number and the topography. Hence, we conclude that the radiative or non-radiative energy transfer from excited $NaGdF_4$ LNPs was not responsible for the observed nonlocal CL signal. The CL signal was most likely due to free space propagation of scattered electrons.

Q2. As mentioned in my previous comment, I still believe describing the relationship between particle diameter and photon detection rate in Figure 4A is inappropriate as a linear (or monotonic increase). Specifically, all the y-intercepts in Figure 4C are negative, significantly deviating from the linear trend observed in Figure 1C or Figure S25C. I would appreciate further clarification from the authors regarding this discrepancy. A proper explanation should be provided if this trend is due to a detection limitation or other experimental constraints.

We apologize for not fully clarifying this point in the previous round of revisions. We agree with the reviewer that the data presented in Figure 4A shows a negative y-intercept for all dopants. This arises from the detection limit of our system, which is primarily governed by the background CL signal from the Si substrate.

An SNR of 3 (SI Fig. 31) or >160 photons/s (SI Fig. 30 & Fig. 4A) were required to reliably detect LNPs. In our system, this detection threshold corresponded to a particle size of roughly 15 nm, although the exact value varies slightly across different dopants (SI Fig. 31). Because CL signal above the background is reported, this minimum detectable size results in a non-zero x-intercept in the plot, which in turn leads to a negative y-intercept in the linear fit.

We have now clarified this point in the main text. In addition, we have addressed the discrepancy between size measurements obtained from SEM versus TEM, which may also contribute to this observation. We hope this revision addresses the reviewer's concerns and clarifies our results. (Main text: 335-39 and 342-45)

Reviewer #4 (Remarks to the Author):

This reviewer thanks the authors for their significant work to address the various comments by reviewers. The majority of comments and suggestions have been adequately addressed. Particularly, the figures have significantly improved and are better connected to the narrative text.

We thank the reviewer for their encouraging remarks on our work in addressing the reviewers' comments from the last round of revisions, and improvement in the figures and their connectivity to the text.

However, this reviewer is still troubled by the stated novelty of the work presented here. Other than not analyzing particle aggregates, what is actually developed?

Our work introduces critical advancements in sample preparation, nanoparticle engineering, spectroscopy, CL instrumentation, imaging, and image processing. These advancements led us to achieve the longstanding goals of multicolor single-particle CL imaging at the nanoscale, single-particle CL rate measurements as a function of particle size, and biological compatibility of single-particle CL emission. Below, we outline these contributions:

Identifying the critical limitation in achieving nanoscale, multicolor CL imaging: Multicolor CL imaging at the nanoscale has been a longstanding goal, with several groups attempting to achieve it over the past 10–15 years[1–8]. In this manuscript, we demonstrate that the primary factor limiting progress has been the presence of nonlocal CL signal caused by stray electrons originating from the excited nanoparticle. Through a combination of simulations and experiments—including varied sample geometries and substrates—we consistently identify stray electron excitation as the underlying mechanism of this artifact that has never been experimentally confirmed or systematically analyzed. This insight clarifies why the nonlocal signal misleadingly appears spatially localized to the excited particle, making it difficult to distinguish from true CL emission. Our findings also explain previously unexplained observations in the literature, such as the signal's independence on particle size[4,7] and the appearance of a single nanoparticle across multiple spectral channels[7]. (Figs. 1&2)

Mitigating the nonlocal signal: After identifying stray electrons as the primary cause of the nonlocal CL signal, we identified and eliminated various sources of CL that could be excited by these electrons. These sources are discussed below:

(i) Removing particle aggregates from samples: Stray electrons from the excited NPs could travel hundreds of micrometers and excite aggregates outside the imaged region but within the field of view of our parabolic mirror. As a result, signals from aggregates, despite not being analyzed/imaged by the electron beam, appeared at the spatial location of imaged NPs. Removing particle aggregates mitigated this issue, as evidenced by two key advances:

1. For the first time, we observed a monotonic scaling of CL intensity with particle size, consistent with Monte Carlo simulations. This provides access to nanoparticle engineering for applications in CL imaging, which has not been possible until CL rate from single particles could be measured, and

2. We successfully demonstrated multicolor CL imaging of multiple individual nanoparticles within the same field of view, which shows a proof-of-concept for using LNPs and CL probes in multicolor biological electron microscopy. This has not been previously achieved with such small nanoparticles (20 nm), only with much larger crystals or nanoparticle aggregates.

Together, these achievements represent a breakthrough in nanoscale CL imaging. (Figs. 3&4)

(ii) Demonstrating single-particle CL in biological samples: We found that biological samples exhibit significant auto-CL, which not only increased background signal but also led to the 'emission' of NPs in the wrong spectral channel (SI Fig. 37). Our mechanistic understanding of nonlocal excitation enabled us to recognize and address this issue. By treating samples with the auto-CL quencher OsO_4, we successfully suppressed background emission. We demonstrated that sub-20-nm single nanoparticles remain visible post-treatment, enabling multicolor CL imaging of individual nanoparticles in biological environments. This is, to our knowledge, the first such demonstration and establishes LNPs as robust, biologically compatible molecular probes. (Fig. 5)

(iii) CL instrumentation for single-particle CL imaging: We consistently observed (even after the above-mentioned optimizations) a consistent signal at the spatial location of NPs at shorter wavelengths. We eventually traced this signal to luminescence from the Everhart-Thornley detector's (ETD) scintillator. This diagnosis was only possible due to our understanding that nonlocal excitation could produce a spatially localized signal. By isolating and minimizing this source, we further optimized our imaging system and expanded the usable spectral range of NPs to the entire visible spectrum. (SI Fig. 40)

Engineering CL nanoparticles and further improving CL imaging: We also demonstrate that a higher dopant concentration, above 20%, is necessary to achieve optimal CL signal from single NPs (SI Fig. 5). Such single-particle optimization was crucial for the use of NPs as reliable probes. Previous studies, by analogy with upconverting nanoparticles, used lower doping levels[3–5]. Because of the combination of factors, including nonlocal excitation, the limitations of

underdoped particles were not identified. Our work resolved this issue and open up future possibilities for engineering of lanthanide NPs for nanoscale CL imaging.

In addition to optimizing particle composition, we identified the optimal imaging parameters—accelerating voltage, beam current, dwell time, drift correction—to reliably achieve single-particle sensitivity in CL. These advancements were crucial for our efforts. For example, if imaging was using a single frame as is typically done instead of many frames that were later aligned (Fig. S21), we would not have been able to get our results.

Thus, our work describes multiple significant technical innovations and findings, advancing the field of nanoscale CL imaging. Multicolor CL imaging using lanthanide NPs has been a long-standing goal, yet it could not be achieved for many years. Our study identifies critical barriers and demonstrates, for the first time, how overcoming them enables single-particle multicolor CL imaging with high fidelity.

Given these contributions, we believe that characterizing our work merely as 'not analyzing particle aggregates' overlooks the fundamental insight and the wide-ranging impact of our findings. We therefore respectfully ask the reviewer to reconsider their assessment in light of the broader implications and technical advancements presented in this study.

It seems that any commercial CL system can produce the same results as long as particles are not aggregated and system conditions are appropriate (e.g. ETD bias voltage, which is system specific due to manufacturers and chamber geometry).

We agree that a commercial CL system could likely reproduce our results, provided that the necessary optimizations are implemented. In fact, before building our own CL system, we had considered purchasing a commercial system in late 2019 to early 2020. However, we encountered two main limitations of the commercial options. First, the commercial systems only had a single spectral channel available at a time, while we needed simultaneous readout in multiple channels (our system supports simultaneous measurements across three channels). Second, the detectors that were available with commercial systems were not sufficiently sensitive in the visible spectral range, particularly towards the red, which would have prevented us from detecting single nanoparticles. These two limitations, combined with the availability of time but limited resources during the COVID-19 pandemic, motivated us to develop a custom system. An alternative would have been to custom-order a similar system from a commercial supplier. However, we chose to build our own system, which provided us the versatility and customizability to pursue the described work (for example, we were able to remove the CL signal from the SE2 detector by introducing a lens tube, which had not been part of the typical design and may have not been possible in a commercial system, SI Fig. 40). We acknowledge that commercial CL systems may have advanced since 2020, and our assessment may not reflect the current state of the field.

By following the strategies outlined in this work, our results can be reproduced with commercial systems, albeit only those with the necessary detection capabilities. We view this not as a limitation but rather as a strength of our work—demonstrating that our approach is transferable

and broadly applicable across systems, and that other groups can take advantage of it. In fact, we lay out the necessary conditions to demonstrate single-particle sensitivity in single-color and multicolor CL imaging:

'To reliably establish single-particle detection sensitivity in both single and multicolor CL imaging, we identified two necessary conditions: a monotonic increase in CL rate as a function of LNP size, and multicolor single-particle imaging in the same field of view.' (Main text: 439–42)

We identified the limitation in achieving multicolor, single-particle CL imaging and discussed strategies to overcome them. However, the required optimizations go well beyond simply avoiding particle aggregation. Even in a (theoretical) sample containing a *single* NP in the entire sample, nonlocal excitation could arise from sources such as auto-CL from biological materials or luminescence from system components (e.g., detectors), and will be localized to the position of the NP. Again, the fact that nanoscale multicolor CL imaging of single particles has not been achieved prior to this work, despite efforts from multiple groups to achieve it, indicates that this paper is a result of more than one or two obvious changes to a sample preparation protocol. This highlights the importance of our work in identifying and systematically mitigating the root causes of nonlocal CL signals.

While parameters, such as ETD bias voltage, are indeed system-specific, isolating luminescence from the imaging system's components is crucial for true single-particle imaging. Otherwise, we could image the spectrum of the ETD at the spatial location of the NP. (SI section 26 and SI Fig. 40)

In summary, our study is the first to uncover and experimentally validate the underlying mechanisms responsible for spectral crosstalk in nanoscale CL imaging. This lack of understanding had previously limited progress in achieving multicolor single-particle CL imaging, despite multiple attempts by other groups. By addressing these foundational issues, our work lays out a clear pathway that enables any properly optimized commercial system to achieve reliable multicolor single-particle CL imaging.

The paragraph at the end of the manuscript beginning with "We achieved single-particle sensitivity by eliminating LNP aggregates from the samples,..." highlights this main concern that persists from the original manuscript. Reducing aggregates does not seem like a development related to CL analysis, but a common sense approach to minimizing signal spread from particle clusters.

The sentence referred to by the reviewer was originally included in a specific context: addressing exciton diffusion through the substrate. It was not meant to support our central claim, and was certainly not intended to be a summary of the entire paper. We have now revised that section to prevent any misinterpretation.

Previous: '*We achieved single-particle sensitivity by eliminating LNP aggregates from the samples, which ruled out the alternative hypothesis that nonlocal CL signal (Fig. 2) was caused by excitons diffusing in the Si substrate rather than stray electrons traveling through free space.*'

Updated: *'The results presented in Fig. 3 demonstrate single-particle sensitivity in dense but non-aggregated samples. These observations, combined with the results presented in Fig. 2E–I, ruled out the alternative hypothesis that the nonlocal CL signal was caused by excitons diffusing in the Si substrate rather than stray electrons traveling through free space.' (Main text: 258–261)*

For an overview of our work, we refer the reviewer to the Conclusion section, which thoroughly discusses the broader optimization strategies that were central to achieving reliable single-particle multicolor CL imaging. (Main text: 443–455)

Again, reducing aggregates is not the only development presented in our manuscript. Rather, the key insight lies in identifying the mechanism of and mitigating nonlocal CL excitation. Specifically, we demonstrated the role of stray electrons originating from an excited nanoparticle that can travel significant distances and excite other CL-active materials, such as aggregates or auto-CL sources, even outside the imaged region. Removing aggregates is only one of the important steps we took to make this imaging possible, and on its own does not necessarily offer single-particle sensitivity because nonlocal signal can occur even in the absence of aggregates as described above.

While in hindsight this mechanism may appear intuitive, it had not been previously demonstrated experimentally or systematically analyzed, despite significant efforts in the field to achieve nanoscale multicolor CL imaging in the same field of view that had not been successful. We believe that our work is the first to provide a mechanistic explanation for this form of crosstalk, and to present practical solutions to overcome it, perhaps even in other CL instruments, beyond our own customized SEM.

We hope this clarification addresses the concern and highlights the broader technical and scientific advancement our study contributes to CL imaging.

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