Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In this manuscript Sobolev et al. present results from the first single particle imaging (SPI) experiments at the European XFEL (XFEL) and show that the MHz intra-pulse train repetition rate at XFEL can be used for such experiments. The use well known model systems for SPI such as Mimi-Virus, which is sufficient for the initial experiments to assess what is even possible at this new facility.

The results are presented in a clear, convincing and technically sound way and I recommend publication with minor revisions:

a)Pg6 Fig2: no description for panels c/d in caption
b)Pg7 Fig3: "Note that the scale is linear below 10−3 photons per pixel" please re-format font. the -3 in 10-3 is almost not readable.
c)Pg9, Fig5 "Single strong diffraction pattern of an IrCl sphere of 439 nm in diameter, edge resolution is 12.7 nm. b
comparison between the radially averaged scattering of the IrCl sphere (orange), fitted model (blue) and radially
averaged background with injection (green). Note that the scale is linear below 10-2 photons per pixel. The red dashed
lines (18.4 nm resolution) mark the angle at which the modeled scattering is stronger than the noise in a single frame;
the purple dashed lines (12.7 nm resolution) mark the angle where the modeled scattering exceeds an average
background; detector edge resolution is 6.5 nm." Which is now the edge resolution? 12.7nm or 6.5nm?
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of the variation of the center of the diffraction patterns show an order of magnitude lower instability than similar measurements at the LCLS AMO instrument (Loh et al, 2013)," and how does it compare to CXI?
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Where Fangohr should be listed after Ekeberg.

Reviewer #2 (Remarks to the Author):

The manuscript 'Megahertz single-particle imaging at the European XFEL' by Sobolev et al. is one of a series of publications on feasibility studies of high-repetition rate imaging experiments at the European XFEL (Wiedorn et al. 2018; Grünbein et al. 2018, Yefanov et al. 2019). The XFEL community as a whole has been anxiously awaiting the advent of high-repetition rate instruments for a number of years, as the first generation was/is limited in terms of throughput. While the manuscript is in itself limited in terms of quantifiable 'results' (i.e. a reconstruction), it describes an important turning point for the field and I would recommend the publication of this manuscript. However, I have several major points that I would like to see addressed prior to publication.

One of the main problems I see with this study is the overall experimental setup, which was presumably not chosen by the authors, but was imposed by what was available at the beamline. The energy of 9 keV in combination with a 15 µm focus would not be my 'experiment of choice' for such an experiment, which ultimately leads not only to limited signal levels, but also missing low q information, making a reconstruction impossible. From what I can tell, the energy of 9 keV is only mentioned in the discussion and given how important of a factor it is, it should be stated earlier. However, I might be mistaken and the energy was deliberately chosen. If that is indeed the case, you should list reasons why. It is also not immediately obvious at what repetition rate the experiments were actually carried out. In summary I would like to see a bit more discussion about the experimental setup: state what limitations lead to this setup and maybe comment on what the ideal setup would have been.

One of the main selling points of this paper is the fact that high-repetition rate data collection is necessary to make single particle imaging possible. However, there is not a single estimate as to how many diffraction patterns would be necessary for a reasonable reconstruction. While I certainly agree that this ultimate goal of high resolution imaging of single particles with X-rays would revolutionize imaging in general, it would be important to expand the discussion as to what needs to happen to achieve it.

Specific points:

- Page 6: IrCl. If a 0.1% solution of IrCl (3 mM) crystallizes during injection with the aerosol injector, it would be important to see what happens with biomolecules in their buffer solutions (~150 mM NaCl) - are they coated with a crust of salt crystals? While this manuscript is not about sample injection, this certainly raises questions.
- Page 7: Background scattering. The information content is relatively limited. It would be helpful to at least state, whether these background levels were anticipated and how they compare with signal levels from a sum of/individual diffraction pattern(s).
- Page 7: Diffraction pattern centers. Similar situation. Describe how this spread would affect the downstream analysis and what are the limits one could compensate for at a given resolution.
- Page 10: While the distribution of particle sizes is certainly worth discussing, I am not convinced that this spread is realistic for a biological sample. Highly symmetric viruses are typically very rigid and have tight constraints on their assembly and if these are not met the virus 'falls apart'. It would be worth comparing these numbers to other measurements, such as cryoEM or DLS, to ensure that the sigma in these curves is not simply representing the experimental error.
- Add an average/sum of diffraction patterns to estimate the 'overall signal levels/resolution' of the experiment from all samples.
- Hitfinding is understandably biasing towards the 'stronger' diffraction patterns. It would be interesting to see the statistics of the weaker 'hits' as well.

Reviewer #3 (Remarks to the Author):

The conclusion of the manuscript that single-particle imaging experiments can be performed at the megahertz intra-bunch repetition rate of the European XFEL is substantiated by this report. The

work provides a number of useful results about the performance of this machine, a relatively new facility that is expected to be used in a wide range of science areas, that will be helpful to persons planning experiments. It extracts:
i) statistical information about the intensity distribution from calibration samples of known density,
ii) the beam pointing variations from the centers of the diffraction patterns,
iii) some information about how to prepare "standard" CDI samples by salt evaporation, and
iv) the size distributions generated by this method.

It also shows that correct size distribution about a known biological sample, a mimivirus, can be produced, consistent with prior knowledge. It does not claim to have produced new information about the salt particles or the virus, but more about the beam properties of the new public facility. I fully support publication of the work for the benefit of those scientists planning to use the European XFEL.

I have a few minor comments about the presentation of the work in the submitted manuscript, which should be considered by the authors:

1. If the Ir is in the usual 3+ state, the short notation IrCl3 should be used for the material, rather than IrCl, which might alarm a chemist.

2. It is correct to give flux numbers as photon density, rather than trying to factor out the beam size to get a raw photon number. However, a rough number for this might be useful to the reader.

3. Boutet et al (2012) showed that the scaling of signal to incident flux for protein crystals did not change with pulse length. That result is implicitly assumed in the interim before the European XFEL pulse length is known. This should be stated as an assumption.

4. The symbol q should not be used for momentum transfer as defined on p7 (pdf). 2\pi is missing from that formula. What is defined there is usually call s, not q.

5. I do not think the water of crystallization is important in calibration the flux from the particle size: only the density should matter. The scattering signal is proportional to the number of electrons and that is known from the volume and density.

Megahertz single-particle imaging at the European XFEL

Egor Sobolev et al.

Changes to the manuscript are colored in orange.

Response to reviewers

Answers to reviewer 1:

(original text from the referee in blue)

In this manuscript Sobolev et al. present results from the first single particle imaging (SPI) experiments at the European XFEL (XFEL) and show that the MHz intra-pulse train repetition rate at XFEL can be used for such experiments. The use well known model systems for SPI such as Mimi-Virus, which is sufficient for the initial experiments to assess what is even possible at this new facility.

The results are presented in a clear, convincing and technically sound way and I recommend publication with minor revisions:

a)Pg6 Fig2: no description for panels c/d in caption

This has now been added.

b)Pg7 Fig3: "Note that the scale is linear below 10−3 photons per pixel" please re-format font. the -3 in 10-3 is almost not readable.

Fixed.

c)Pg9, Fig5 "Single strong diffraction pattern of an IrCl sphere of 439 nm in diameter, edge resolution is 12.7 nm. B comparison between the radially averaged scattering of the IrCl sphere (orange), fitted model (blue) and radially averaged background with injection (green). Note that the scale is linear below 10-2 photons per pixel. The red dashed
lines (18.4 nm resolution) mark the angle at which the modeled scattering is stronger than the noise in a single frame; the purple dashed lines (12.7 nm resolution) mark the angle where the modeled scattering exceeds an average background; detector edge resolution is 6.5 nm." Which is now the edge resolution? 12.7nm or 6.5nm?

We agree this was confusing. The edge resolution of 12.7 nm is actually the edge resolution of the image shown, which is a crop of the full detector. The full detector has an edge resolution of 6.5 nm. We have now changed the caption to avoid this confusion.

d)pg13 "Our measurements of the variation of the center of the diffraction patterns show an order of magnitude lower instability than similar measurements at the LCLS AMO instrument (Loh et al, 2013)," and how does it compare to CXI?

It's also about an order of magnitude below CXI. This has now been added to the text.

e)pg13 "Using electrospray instead of GDVN for the formation of the aerosol is likely to eliminate this problem" please rephrase to "Using electrospray instead of GDVN for the formation of the aerosol could solve this problem"
Done.

f)Wouldn't it be good to use the collected data on Mimivirus and continue with data processing to compare results with previous work by Seibert et al and Ekeberg et al? The amount of positively identified single hits for Mimi-Virus should be sufficient for this…
The main issue is the relatively poor resolution of the data, compared to Seibert et al and Ekeberg et al. This is due to the larger than expected focal spot on this experiment, as well as the lower cross-section for harder X-rays. We hope to carry out such comparisons in future experiments.

g) Citations: are not always in alphabetical order in the bibliography.
"Daurer, B. J. et al. Experimental strategies for imaging bioparticles with femtosecond hard Xray pulses. IUCrJ 4, 251–262 (2017).
Fangohr, H. et al. Data analysis support in Karabo at European XFEL. In Proc. 16th Int. Conf. on Accelerator and Large Experimental Control Systems, TUCPA01, 245-252 (Barcelona, Spain, 2017).
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Ekeberg, T. et al. Three-Dimensional Reconstruction of the Giant Mimivirus Particle with an X-Ray Free-Electron Laser. Phys. Rev. Lett.114, 98102 (2015)."

Where Fangohr should be listed after Ekeberg.
Thank you for pointing this out. It has now been corrected.

Answers to reviewer 2:
(original text from the referee in blue)
The manuscript 'Megahertz single-particle imaging at the European XFEL' by Sobolev et al. is one of a series of publications on feasibility studies of high-repetition rate imaging experiments at the European XFEL (Wiedorn et al. 2018; Grünbein et al. 2018, Yefanov et al. 2019). The XFEL community as a whole has been anxiously awaiting the advent of high-repetition rate instruments for a number of years, as the first generation was/is limited in terms of throughput. While the manuscript is in itself limited in terms of quantifiable 'results' (i.e. a reconstruction), it describes an important turning point for the field and I would recommend the publication of this manuscript. However, I have several major points that I would like to see addressed prior to publication.

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The energy of 9 keV in combination with a 15 µm focus would not be my 'experiment of choice' for such an experiment, which ultimately leads not only to limited signal levels, but also missing low q information, making a reconstruction impossible. From what I can tell, the energy of 9 keV is only mentioned in the discussion and given how important of a factor it is, it should be stated earlier. However, I might be mistaken and the energy was deliberately chosen. If that is indeed the case, you should list reasons why. It is also not immediately obvious at what repetition rate the experiments were actually carried out. In summary I would like to see a bit more discussion about the experimental setup: state what limitations lead to this setup and maybe comment on what the ideal setup would have been.

We agree with the referee that we should have explained the reason for the parameters chosen. Those were the only parameters available for the first run of user experiments. As the referee correctly points out they are far from optimal. We now state the key experimental parameters such as photon energy, focal spot and repetition rate much earlier, on page 4. In the discussion we also mention why they were used and what would be the ideal parameters.

One of the main selling points of this paper is the fact that high-repetition rate data collection is necessary to make single particle imaging possible. However, there is not a single estimate as to how many diffraction patterns would be necessary for a reasonable reconstruction. While I certainly agree that this ultimate goal of high resolution imaging of single particles with X-rays would revolutionize imaging in general, it would be important to expand the discussion as to what needs to happen to achieve it.

An accurate estimate of the number of patterns required for a given resolution is not trivial given the large number of parameters that influence it. There are some theoretical calculations, which don't include problems such as sample heterogeneity and tend to give excessively optimistic results. There are too few experimental results to form the basis of a robust extrapolation. We've expanded the discussion to tackle this issue and to point out other important improvements necessary for high-resolution single-particle imaging with X-rays.

Specific points:

- Page 6: IrCl. If a 0.1% solution of IrCl (3 mM) crystallizes during injection with the aerosol injector, it would be important to see what happens with biomolecules in their buffer solutions (~150 mM NaCl) - are they coated with a crust of salt crystals? While this manuscript is not about sample injection, this certainly raises questions.

The viruses were dialyzed five times in 250 mM ammonium acetate, as pointed out in the sample preparation, to eliminate their native buffer. So the concentration of native buffer in the injected solution is negligible. If we had not done so, then there would certainly be a crust of buffer around the sample.

- Page 7: Background scattering. The information content is relatively limited. It would be helpful to at least state, whether these background levels were anticipated and how they compare with signal levels from a sum of/individual diffraction pattern(s).

In the discussion, we mention that the background levels are better than at CXI beamline of the LCLS, so somewhat better than what was expected. We also have a comparison between the background and the signal in fig. 5.

- Page 7: Diffraction pattern centers. Similar situation. Describe how this spread would affect the downstream analysis and what are the limits one could compensate for at a given resolution.
We briefly refer to this in the discussion, by saying that the variation in the center is well below a Shannon pixel. We have now expanded on this, hopefully making the consequences clearer for the reader.

- Page 10: While the distribution of particle sizes is certainly worth discussing, I am not convinced that this spread is realistic for a biological sample. Highly symmetric viruses are typically very rigid and have tight constraints on their assembly and if these are not met the virus 'falls apart'. It would be worth comparing these numbers to other measurements, such as cryoEM or DLS, to ensure that the sigma in these curves is not simply representing the experimental error.
We agree with the referee that the wide distribution of particle sizes does not correspond to the size distribution in physiological conditions, and is likely due to contaminations, e.g. from unassembled viral particles as the referee points out. We have added a sentence to final part of the discussion making this clear.

- Add an average/sum of diffraction patterns to estimate the 'overall signal levels/resolution' of the experiment from all samples.
We have added an average of the signal of all hits for each sample type as figure 6.

- Hitfinding is understandably biasing towards the 'stronger' diffraction patterns. It would be interesting to see the statistics of the weaker 'hits' as well.
The statistics for the weak hits will always have a very large error due to the low signal strength in them as well the reduced number of events, which is why we opted not to 'slice' the statistics in this way.

Answers to reviewer 3:

(original text from the referee in blue)

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It also shows that correct size distribution about a known biological sample, a mimivirus, can be produced, consistent with prior knowledge. It does not claim to have produced new information about the salt particles or the virus, but more about the beam properties of the new public facility. I fully support publication of the work for the benefit of those scientists planning to use the European XFEL.

I have a few minor comments about the presentation of the work in the submitted manuscript, which should be considered by the authors:

1. If the Ir is in the usual 3+ state, the short notation IrCl3 should be used for the material, rather than IrCl, which might alarm a chemist.
We have fixed the notation.

2. It is correct to give flux numbers as photon density, rather than trying to factor out the beam size to get a raw photon number. However, a rough number for this might be useful to the reader.
As the sample only interacts with a small cross-section of the beam it is not possible to convert from the photon density to the number of photons in the beam. We have added to the manuscript the total number of photons measured by the gas monitor detector in the "Experimental set-up at the SPB/SFX instrument" section.

3. Boutet et al (2012) showed that the scaling of signal to incident flux for protein crystals did not change with pulse length. That result is implicitly assumed in the interim before the European XFEL pulse length is known. This should be stated as an assumption.
We agree with the referee that we are assuming that the X-ray beam is not changing the response of the sample. We have now stated this explicitly on page 5.

4. The symbol q should not be used for momentum transfer as defined on p7 (pdf). 2\pi is missing from that formula. What is defined there is usually call s, not q.
We have replaced q with S.

5. I do not think the water of crystallization is important in calibration the flux from the particle size: only the density should matter. The scattering signal is proportional to the number of electrons and that is known from the volume and density.
The number of electrons cannot be exactly known without knowing the elements in the sample, as different elements have different proton to neutron ratios (and consequently different atomic mass to atomic number ratios), but it is true that a very good approximation can be made. We also noticed there was a mistake in the number of water molecules, which has now been corrected. There was no appreciable change to the results, which is consistent with the argument of the referee.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

Thanks for replying to my comments. The article can now be published as is.

Reviewer #2 (Remarks to the Author):

I have no further comments and suggest the publication as is.