Appendix S4: Methodological considerations for viral nanoSIMS and viral BONCAT

1. Methodological challenges and considerations for viral isotope measurements

Due to the low biomass per viral particle, there are some current limitations for nanoSIMS to provide quantitative data for individual viral particles. The absolute level of enrichment is confounded by high error in viral isotope measurements as well as anticipated variability in host label uptake resulting either from growth rate differences between hosts and/or temporal variability between label uptake and lysis events. Therefore, the power of isotope tracer experiments is in obtaining quantitative estimates for the total number of viruses that have been produced directly or indirectly from primary productivity or a particular nutrient or carbon source, rather than providing a direct comparison of the enrichment levels between host and virus. Furthermore, error sources beyond the counting statistics alone – e.g., biological variability, variable analytical background contributions to the small biomass, and beam distortions and frame alignment – play an important role and should be considered when evaluating quantitative differences between individual virus particles.

Given the challenging degree of inherent analytical uncertainty, rigorous sample preparation also becomes particularly crucial for effective nanoSIMS analysis of viral particles. We recommend depositing viruses onto a conductive flat surface, adding similar size particles with a unique ion signature in with the viruses to aid in frame alignment, and considering the impact of the level of isotopic label and low count rates on isobaric interferences, particularly for dual labeling experiments. While we have identified the importance of these methodological considerations for effective analysis of viral particles on the nanoSIMS, future efforts are necessary to continue developing the best practices for viral nanoSIMS.

1a. Silicon wafers – a conductive flat surface

We recommend using silicon wafers, which provide a conductive flat surface and therefore do not require sputter coating (Orphan and House 2009). Gold coating, which is

commonly done for analysis of samples placed on glass slides, creates a challenge for analysis of viral particles due to the need to pre-sputter. High beam current pre-sputtering would destroy the viral particles of interest. Furthermore, adding precise fiduciary marks to the silicon wafers using a laser dissecting microscope (LMD) was critical for locating the samples on the nanoSIMS.

1b. Addition of gold particle nanoparticles

Accurately quantifying counts from several atomic masses originating from the same pixel location is what enables the derivation of meaningful isotope ratios from the nanoSIMS. Over longer acquisition times there can be slight drift from one frame to another (by several pixels). While this drift is typically corrected for when multiple frames are merged (Polerecky et al. 2012), the ability to correct for drift depends on features visible in the ion images. Aligning images offset by only a few pixels (which is essentially the size of the viral particles) presents an analytical challenge when each pixel only has a few counts. Microbial cells typically analyzed by nanoSIMS span a much larger area than a few pixels making frame alignment easier. For the majority of our analyses, we used $^{14}N^{12}C-$ to locate the viral particles on the nanoSIMS as well as align the images. Nitrogen is useful for this not only because $^{14}N^{12}C-$ is a good secondary ion, but also because viral particles are enriched in nitrogen relative to carbon when compared to microbial cells (Jover et al. 2014). We tested gold particles (that were of a similar size range as the viral particles) as a means to improve our ability to merge ion images across multiple frames, particularly for Syn1, the smallest virus we analyzed. While we were still able to obtain meaningful data from Syn1 without gold particles because of the presence of detrital particles in each frame, the addition of gold particles will prevent the need to discard frames without detrital particles and make the analysis of even smaller viral particles possible. Furthermore, because the viral particles are not visible on the nanoSIMS CCD camera or in SEM mode, reference markers such as the gold particles seem promising in terms of helping locate target regions for analysis.

1c. Separation of Isobaric Interferences

Separation of isobaric interferences (ions with the same nominal atomic mass) is an important aspect of the nanoSIMS that enables high mass-resolution detection of specific ions (Slodzian et al. 1992). Examples of isobaric interferences relevant for C and N isotope analysis include $^{14}N^{12}C^-$, $^{13}C_2^-$ and $^{12}C^{13}CH^-$ as well as $^{15}N^{12}C^-$, $^{14}N^{12}CH^-$ and $^{14}N^{13}C^-$ (Burdo and Morrison 1971). Dual labeling with both ^{15}N and ^{13}C may cause bigger than expected isobaric interferences, because some of these otherwise rare ions (e.g., $^{13}C_2^-$ and $^{12}C^{13}CH^-$) may be much more prevalent. Separation of isobaric interferences can be significantly improved by modifying the entrance and/or exit slits (Slodzian et al. 2014). However, these modifications can often result in reduced ion count rates (10-20%). While this is not necessarily problematic for larger microbial targets, altering these tuning conditions presents problems for viral targets, which already have low ion counts. While we employed high levels of isotope label (particularly for N) during our proof of concept experiments, lower levels of label tracer enrichment (10-20%) are recommended for environmental studies in the future. This level would still be detectable with the nanoSIMS, but reduce the tuning challenges and signal suppression due to isobaric interferences.

2. Methodological challenges and considerations for measuring viral activity via BONCAT

There are several important methodological considerations for the application of BONCAT in the marine environment. This approach depends on the assumption that phytoplankton and heterotrophic bacterial hosts incorporate HPG and subsequently transfer HPG to their viral particles with enough HPG to generate a fluorescence signal above background. Previous publications and work have confirmed that heterotrophic bacteria, cyanobacteria and eukaryotic phytoplankton can take up HPG, but the magnitude and rate of uptake can vary (this study, Hatzenpichler et al 2014, 2016, Samo et al. 2014; Figs. 5F, 6F). Since variation in host uptake may influence viral incorporation of HPG, the ability of different marine microorganisms to utilize HPG and transfer this label to their viruses needs to be tested further. For the model systems we studied, viral methionine content (at least for T7, Syn1 and EhV) is about 3% of the total amino acid pool (Table S1), similar to that of bacteria and archaea (Hatzenpichler et al. 2014, Samo

et al. 2014). Therefore, the number of methionine residues within the viral proteins indicates that every protein has the potential to become HPG-labeled. The labeling efficiency is also dependent on the HPG to methionine ratio in the incubation as well as the selectivity of the respective methionyl-tRNA synthetase (Kiick et al. 2002). The methionine to HPG ratio has been shown to influence HPG uptake (Samo et al. 2014). In environments where cells are very active, continuous HPG additions may be needed to obtain sufficient labeling of the host populations (and viral particles) over time. This is one possible explanation for why we saw a reduction in the number of HPG-labeled viral particles between 48 and 72 hours. Another explanation is of course that the host cells from which those viruses were produced did not take up HPG.

3. References

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