Dear editor,

We are grateful for the careful review by editor and reviewers. The requested changes have been included in a revised manuscript. We find that the quality of the manuscript has substantially increased by the review process.

Sincerely,

Niels de Jonge

**Detailed response to the reviews**

**Editorial comments:**

-Formatting: -3.1.11 – The paragraph containing the equation should be a note. -

Response: has been corrected.

-Please break 3.3.4 into two steps.

Response: has been done.

 -3.2.5 – Wrong step is cited.

Response: has been corrected into: “in steps 3.1.7 – 3.1.9”

-References – Please abbreviate all journal titles.

Response: has been done.

•Grammar:

-Please copyedit the manuscript for numerous errors, some of which are noted below. Such editing is required prior to acceptance and should be performed by a native English speaker especially to correct awkward phrasing.

-Line 31 – “at the nanoscale” what?

Response: corrected into: “with nanoscale spatial resolution”

-Line 117 – “The fume hood is used for chemical safety but if the laboratory regulations allow this it is preferred from the point of view of avoiding dust collection to do all work in the laminar flow hood” – very awkward phrasing

Response: changed into: “Note: The steps involving acetone are performed in a fume hood for chemical safety.”

-1.1.8 – “alumina foil”

Response: changed into: “aluminum foil”

-2.1.6, Line 2.3.9 – “a tweezers”

Response: changed into “tweezers”

-3.1.6, 3.1.3, 3.2.5, 3.2.6, Results section (and figure legends) – “20kx”; “400kx” – this is not a typical way to express this in a formal manuscript.

Response: changed into: 20,000× etc

-Line 382 – “This value is much larger than based on the spacer thickness” – awkward phrasing

Response: sentences changed into: “…0.5 µm, much larger than what was expected based on the spacer thickness of 200 nm. Nevertheless, the thickness is not too large for the imaging of gold nanoparticles with nanometer spatial resolution.”

-Line 476 – “the experiments outcome”

Response: changed into: “the outcome of the experiments”

-Line 460 – “AuNPs. Of which several are selected with arrows.”

Response: has been corrected into: “AuNPs, of which several are selected with arrows.”

-Line 538 – “But it is of course not possible to flow liquid in these systems.” – Do not begin sentences with a conjugative.

Response: changed into: “However, it is impossible to flow liquid in those systems.”.

•Additional detail is required:

-1.1.7, 1.18 – Is this done by hand or using a shaker?

Response: inserted: “2by hand”

-2.2.10 – What kind of enclosure would this be?

Response: changed into: “its enclosure”. Note: Each commercial holder comes with an enclosure.

-3.1.5 – Where/how is the ADF detector inserted?

Response: included: “…by pressing the ADF button.

Note: this protocol refers to an ADF detector positioned above the phosphor screen of the microscope.”

-3.1.7 – How are these settings adjusted?

Response: included: “(using the respective knobs)”. The microscope has contrast and brightness knobs.

-3.1.9 – How is the position stored?

Response: included: “using the store button of the software.”

-3.1.10 – The magnification was already at 20000X in 3.1.6. When was it changed?

Response: deleted: “Increase the magnification to 20,000x”.

-3.1.11 – What is the expected thickness? How is the current density measured/viewed?

Response: changed into: “Proceed only if the liquid thickness has been determined and does not exceed 3 µm.” and “Make note of the current density measured at the phosphor screen visible via the operating software…”

-3.1.13 – Please elaborate on “interesting” and “satisfying images.” What should one be looking for?

Response: changed into: “…an area containing at least 20 nanoparticles.” and “Once images have been obtained in which the gold nanoparticles are visible with strong contrast and sharp edges (see Figure 6),”

•Branding: 2.1.3 – Teflon

Response: changed into: “polymer”

•Results:

-Line 397 – Please delete reference to “online Movie 1” which has not been provided.

Response: was deleted.

-Figure 7 – Please describe each individual panel in the figure legend.

Response: not useful to define each panel as those are frames of a time lapse series. For clarity, legend text was changed into: “Time-lapse series of STEM micrographs of AuNPs in saline. A-D: Images extracted from the time-lapse series of STEM images at 30 sec intervals…..”

•Discussion: Please discuss the future applications of the protocol.

Response: the following paragraph was added at the end of of the discussion: “Liquid-phase TEM and STEM are not yet routine analytical techniques but are still developing. The number of parameters to take into account is considerable, and it is often difficult to reproduce experimental results. Moreover, quantitative data is difficult to obtain because the effects under investigation are intertwined with processes occurring as a result of the electron beam. The protocol described here aims to standardize the experimental protocol, thereby accounting for all relevant base aspects of the experiment. We hope that this protocol will lead to better reproducibility of experimental work in this emerging field.”

•Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

Response: has been done.

**Reviewers' comments:**

**Reviewer #1:**

No changed requested

**Reviewer #2:**

2.1. This application note would be more suitable as an application note on the manufacturers website than as a publication. ….this paper it is not stated what is novel/innovative/gold standard (everything has been published before). …. This submission is not a paper reporting scientific novelty value”.

Response: many papers about liquid-phase TEM and STEM have been published but to our experience important detail is lacking in literature to conduct those experiment in a successful manner. Scientists who want to start using this technology require extensive training and it often takes many weeks before the experiments become successful. The technology is not (yet) standard. Even existing users often have problems. A detailed experiments protocol is lacking in literature of how to set about in those experiments. Our paper presents a detailed guideline that provides scientists with the highly needed information to get started in this field in a systematic manner. Thus, we find the paper indeed contains novel and important information not yet published in the open literature.

**Reviewer #3:**

3.1. Page 10 (lines 307-308): put the mean free paths in italic, as in equation (1).

Response: has been corrected.

3.2. The legend of Figure 8; correct the beginning of the legend: "A: SiN membrane with AuNPs. Of which several are selected with arrows.

Response: has been corrected.