**TITLE:**

**Studying dynamic processes of nano-sized objects in liquid using scanning transmission electron microscopy**

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**KEYWORDS:**

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**SHORT ABSTRACT:**

This protocol describes the operation of a liquid flow specimen holder for scanning transmission electron microscopy of AuNPs in water, as used for the observation of nanoscale dynamic processes.

**LONG ABSTRACT:**

Samples fully embedded in liquid can be studied at a nanoscale spatial resolution with scanning transmission electron microscopy (STEM) using a microfluidic chamber assembled in the specimen holder for transmission electron microscopy (TEM) and STEM. The microfluidic system consists of two silicon microchips supporting thin silicon nitride (SiN) membrane windows. This article describes the basic steps of sample loading and data acquisition. Most important of all is to ensure that the liquid compartment is correctly assembled, thus providing a thin liquid layer and a vacuum seal. This protocol also includes a number of tests necessary to perform during sample loading in order to ensure correct assembly. Once the sample is loaded in the electron microscope, the liquid thickness needs to be measured. Incorrect assembly may result in a too-thick liquid, while a too-thin liquid may indicate the absence of liquid, such as when a bubble is formed. Finally, the protocol explains how images are taken and how dynamic processes can be studied. A sample containing AuNPs is imaged both in pure water and in saline.

**INTRODUCTION:**

Conventional scanning transmission electron microscopy (STEM) is limited by the range of specimens appropriate for analysis, specifically the dry and solid samples suitable for placement in a high vacuum. However, many scientific and technological questions concern nanoscale materials and processes in liquid environment. Samples fully embedded in liquid can now be studied with STEM using a concept that involves a microfluidic chamber assembled in the specimen holder for transmission electron microscopy (TEM) and STEM1. This newly developed technique has become increasingly popular, as it provides new insight into important processes of various research topics, including the growth, dissolution, and aggregation processes of nanoparticles2-6. Not only metals, but also biominerals7 and biological systems can be studied8-11. The sample loading and image acquisition for liquid-phase STEM is different than for STEM of dry samples and involve a protocol that requires dedicated training.

The microfluidic system consists of two silicon microchips supporting silicon nitride (SiN) membrane windows transparent for the electron beam at 200 keV of energy12 (see Figure 1A). Details of the dimensions and of the handling of these microchips can be found elsewhere12,13. The sample usually contains nanoscale objects—gold nanoparticles (AuNPs) in this paper. The AuNPs are immobilized at the top window (with respect to a downward-traveling electron beam) or float in the liquid. Nanoscale spatial resolution in STEM is obtained by scanning the electron beam over the AuNPs and collecting transmitted scattered electrons using the annular dark field (ADF) detector9. The two microchips are placed in a small slot in the tip of the liquid flow TEM holder1 (the holder operates for both STEM and TEM but is referred to as the TEM holder). One of the microchips contains a spacer so that a liquid compartment is formed between the microchips. O-rings on both sides of the two microchips provide vacuum sealing of the liquid compartment13 (see Figure 1B).

The aim of this article is to demonstrate the basic steps of sample loading and data acquisition so that interested users may find easy access to this emerging new technique. A system available from a specific company is used, but the protocol is also valid for systems of other companies. The technique is more complex than conventional TEM and STEM, and a number of practical aspects must be considered when working with a liquid holder system13. Most important of all is to ensure that the liquid compartment is correctly assembled, thus providing a thin liquid layer and a vacuum seal. Therefore, it is highly important to work cleanly and to prevent the formation of dust during the preparation and assembly of the liquid flow TEM holder. In particular, the O-rings and the two silicon microchips need to be free from all contamination. Even small particles of dust on one of the microchips may severely increase the thickness of the assembled cell, which may prevent the achievement of a useful spatial resolution. A vacuum seal is important so that no contamination or damage will be left in the electron microscope after the experiment. This protocol describes the loading procedure and several necessary tests. The operation of the electron microscope is straightforward, but it requires some extra steps compared to microscopy of solid samples. With increasing liquid thicknesses, more electrons are absorbed and scattered by the liquid; a measurement of the liquid thickness is essential. Finally, the protocol explains how images are taken and how dynamic processes can be studied.

*[Place Figure 1 here]*

**PROTOCOL:**

*[Place Figure 2 here]*

*[Place Figure 3 here]*

* 1. **Preparation of the microchips**
     1. Prepare a workspace with a low dust level in a laminar airflow hood with a dust particle filter.
     2. Clean a light microscopy glass slide with a fiber-free tissue and pure ethanol for placement and transport of the microchips. Place the glass slide on a cleanroom tissue in a petri dish.

Note: Avoid using technical ethanol at all times.

* + 1. Select 5 base microchips (without a spacer) for sample placement and 5 microchips with a spacer 200 nm thick.

Note: The window dimensions are 20 × 400 µm2 and the SiN thickness is 50 nm. Since there is some tolerance on the dimensions, it is recommended to check the dimensions using a light microscope.

* + 1. Use carbon-coated tweezers to remove the microchips from the storage box and place them on the glass slide. Grab the microchips firmly but carefully on their long sides. Handle the microchip such that the SiN membrane side is always facing upwards.

Note: Avoid touching the fragile SiN membrane on the upper side. Also be careful with handling the microchips to avoid cracking their sharp edges, which can cause problems due to silicon particles residing on the microchip or to later leakages.

Note: Training for the procedure to remove the microchips from the sticky surface can be performed on (cheap) dummy microchips.

* + 1. Place the microchips onto a cleanroom tissue in a petri dish. Close the dish and transfer it to the fume hood.

Note: The steps involving acetone are performed in a fume hood for chemical safety.

* + 1. Take two 120-mL glass beakers. Rinse one beaker with acetone and the other with ethanol to clean them. Fill the first one with 40-60 mL of acetone and the other with 40-60 mL of ethanol.

Note: It is important to use HPLC-grade liquids also for this step in the protocol.

* + 1. Transfer the microchips into the beaker with acetone in order to remove the protective resist layer. Gently move the beaker by hand to effectively rinse the microchips, but be careful not to turn the microchips upside down. See Figure 2.
    2. After 2 min, remove the microchips and quickly transfer them into the beaker with the ethanol. Gently move the beaker by hand with ethanol for 2 min to remove any residues of the resist layer. Cover the beaker with aluminum foil and transfer it to the laminar airflow workbench.

Note: Do not let the microchips dry out during transfer to avoid the deposition of debris.

* + 1. Remove the microchips from the beaker and place them onto a new cleanroom tissue. Let them dry for a few minutes and transfer the microchips onto the light microscope glass slide in the petri dish.

Note: The wet microchips can easily flip around, turn upside down, or stick to the tweezers when being released. This can be avoided by pressing the microchips softly on the filter paper and pulling the tweezers away in the horizontal direction.

* + 1. Close the petri dish and transfer the microchips to the plasma cleaner.
    2. Place the glass slide with the microchips into the plasma cleaner. Run a 5-min cleaning program to render the surface of the silicon nitride membrane hydrophilic and to remove hydrocarbons.

Note: Suitable settings applied for our plasma cleaner are: 70 mTorr, gas flow of 11.5 sccm for O2 and 35.0 sccm for Ar, 50 W forward radio frequency (RF) target, 5 W RF range, and max reflected RF. Any plasma capable of inducing a surface charge is suitable.

* + 1. Put the glass slide with the microchip back into the petri dish, close the lid, and transfer it to the light microscope.
    2. Examine the microchips under a light microscope for possible membrane ruptures or remaining dirt particles. Take special care with the window areas and check for small spots indicating a rupture of the membrane. Dismiss microchips with damaged membranes.
    3. Close the petri dish and store it in the laminar airflow workbench.
  1. **Preparation of the sample on the microchips**
     1. Prepare an aqueous AuNP solution (citrate stabilized) by mixing small amounts of the stock solutions containing 30 nm-diameter, citrate-stabilized AuNPs at a concentration of ~3 M.
     2. Place the microchips on a clean, sticky surface in a transport box for droplet application/sample deposition.
     3. Place a droplet (1-2 µL) of the sample solution on the SiN membrane side of the freshly plasma-cleaned microchip (use a microchip without a spacer) and let the solution dry in the laminar airflow workbench.

Note: This procedure can be repeated in order to increase the concentration of AuNPs on the SiN membrane.

* + 1. Apply 1 µL of deionized water to the microchip for the removal of salt and/or surfactants. After 30 s, carefully remove the droplet of water with filter paper. Let the microchips dry in air in the laminar airflow workbench.

Note: A sufficiently large number of AuNPs will have adhered to the microchip and will not be washed away in water anymore.

* + 1. Use the prepared sample microchips within 4 h, as the SiN loses its hydrophilic rendered surface properties over time, which may cause the liquid to behave differently during an experiment. Note: Refer to the discussion section for more details.
    2. Use a closed transport box for the storage and transfer of the microchips.

1. **Preparation of the liquid flow TEM holder**
   1. **Cleaning the liquid flow TEM holder**
      1. Clean all tools (tweezers and a screw driver) that will be in contact with vacuum parts using acetone and ethanol in an ultrasonic bath and place them on the dust-free surface provided by a piece of new aluminum foil placed on the work bench. Work with gloves to avoid contamination of the liquid flow TEM holder.

Note: The tools do not have to be extensively cleaned if one is sure that they are clean for work with vacuum parts. Gloves can be avoided if one is capable of handling the equipment without touching the vacuum parts.

* + 1. Place the liquid flow TEM holder under the binocular light microscope in such a way that the tip of the holder containing the liquid chamber can be observed. See Figure 3.
    2. Remove the lid of the holder tip and place it on the dust-free surface.

Note: The titanium lid is sensitive to scratches caused by harder materials like the tweezers. It is recommended to use polymer-coated tweezers for sensitive materials.

* + 1. Prepare at least 1-2 mL of pure water (HPLC grade).

Note: If one does not use HPLC-grade liquid, then it is recommended to check that the liquids used do not contain micro particles that could possibly lead to clogs of the flow system; filter the liquid as needed with a micro-pore filter.

* + 1. Use a glass syringe (1 mL) to flush the whole flow system with 0.5 mL of pure water. It is recommended to use a microfluidic syringe pump system. Use a pipette and/or filter paper to remove the liquid being ejected in the liquid cell compartment. Test all tubing for liquid flow. Dry the holder tip afterwards using filter paper and/or a pipette.
    2. Use the light microscope to check that the tip of the holder is clean and dry. If needed, wash the tip of the holder with water or ethanol to remove any solid residues that might have been left behind by the dried solution. Remove pieces of dust or remainders of fibers as well. Carefully remove these pieces with tweezers, thereby avoiding scratching the titanium surface. Also check inside the O-ring grooves and remove the remainders of liquid with a small piece of cleanroom tissue.

Note: If the holder does not become clean with this procedure, then it is recommended to remove the tip from the holder and clean it in acetone for 2 min and in ethanol for another 2 min using an ultrasonic bath.

* + 1. Inspect all further parts of the tip (O-rings, lid, and screws) under the light microscope and remove dust, fibers, etc.

Note: Occasionally, small pieces originating from the screws or the Si microchips must be removed as well. If necessary, briefly clean the vacuum parts with HPLC-grade ethanol. The lid and screws can be cleaned using ultrasound, as explained for the tip. Avoid placing the O-rings frequently in ethanol, as it may make the O-rings brittle over time, diminishing their vacuum tightness. The system is operated without vacuum grease, but if sealing problems occur, vacuum grease can be used.

* + 1. Reinsert the O-rings into the respective grooves of the holder and check that they fit and do not protrude on any side of the groove.
    2. Keep the liquid flow TEM holder free from dust until sample loading (*e.g.,* by covering it with aluminum foil).
  1. **Assembling the liquid flow TEM holder**

Note: The following procedure describes the loading procedure of microchips in a specimen holder with optimal orientation for STEM. In this configuration, the base microchip with the sample will be the upper microchip once transferred into the microscope. See Figure 4. For TEM, the highest spatial resolution is obtained at the bottom of the liquid cell with respect to a downward-traveling electron beam. Thus, the microchips would be mounted the other way around.

* + 1. Use curved tweezers to grab the sample microchip on the long side and place it inside the slot (pocket) in the tip of the liquid flow TEM holder. Keep the SiN side facing upwards. Check the correct placement of the microchip in the slot using a binocular light microscope.

Note: If the O-ring below the microchip is not correctly placed, the microchip may protrude from the slot.

* + 1. Place a droplet of 0.3 µL of the filtered liquid for the experiment on the sample microchip using a micropipette. If necessary, fix the microchip in place with tweezers, as the capillary forces of the droplet may pull the microchip out of its pocket.
    2. Use upside down curved tweezers to take the second microchip (the one with the spacer layer). Carefully turn the microchip upside down. Place the spacer microchip on the base microchip in the slot.

Note: This procedure needs to be done sufficiently quickly to avoid the drying of the droplet on the sample microchip.

* + 1. Inspect the correct placement using a binocular light microscope while moving a piece of light-reflecting material below the tip of the specimen holder. Both microchips must be aligned exactly parallel to achieve the best overlap of the SiN windows.

Note: In case the windows do not overlap, the microchips can be repositioned by pushing at one side with a tip of the tweezers, but avoid moving the microchips too much, as the SiN membrane can be damaged easily. Some microchips come with a crossed configuration (the window of one microchip is perpendicular to the window in the other microchip); this configuration facilitates the alignment of the two microchips yet also reduces the field of view in the electron microscope.

* + 1. Take the front side of the specimen chamber lid with the tweezers and turn it upside down. Without touching the microchips, place the rear side of the lid on the tip. Slowly open the tweezers in such a way that the lid solely rests on the lower branch of the tweezers. Carefully lower the lid until it rests on the two microchips. Remove the tweezers.
    2. Re-check the alignment of the windows of both microchips. If necessary, remove the lid, adjust the positioning of the microchips, and position the lid again.
    3. Place the screws and fix them in their usual places. Check the alignment of the two windows. If necessary, remove the screws again and adjust the microchips.

Note: Do not fix the screws too tightly, as this may cause damage. A vacuum seal is achieved when the screws just tighten.

* + 1. Check the sealing by initiating a liquid flow through the system using a flow rate of 4 µL/min. If no leaking is observed on either side of the tip, this is a good indication of the vacuum tightness.
    2. Transfer the holder to the vacuum pump station and check the vacuum tightness. The vacuum level should reach at least 10-5 mbar within 5 min of pumping.

Note: It is recommended to test the pump station with a dummy holder prior to usage.

* + 1. Transfer the holder to the electron microscope in its enclosure to prevent it from collecting dust.

Note: Each commercial holder comes with an enclosure.

1. **STEM of a specimen in liquid**
   1. **Adjusting the electron microscope for STEM**

Note: The operation of the electron microscope described in this protocol is based on standard procedures that can be found in the user manual. The protocol describes the required detail beyond standard procedures for data acquisition on liquid specimens.

* + 1. Start the microscope with STEM mode aligned. In the specimen holder, insert a test sample consisting of a thin carbon film with AuNPs. Adjust the settings of the STEM microscope as follows: set the probe current to 0.18 nA and the convergence semi-angle of the electron beam to 20 mrad by selecting a spot size of 4C and the objective aperture of 30 µm. Select an 8-cm camera length.
    2. Make note of the indicated current density measured at the fluorescence screen while the ADF detector is inserted. Remove the specimen holder.

Note: This current value is needed later on to estimate the thickness of the liquid.

* + 1. Start the liquid flow with pure water. Don’t exceed a flow of 2 µL/min.
    2. Insert the liquid flow TEM holder in the electron microscope and start evacuation. Check both the vacuum indication of the pre-vacuum chamber and the duration of evacuation. If the vacuum level is standard and the duration of the pumping procedure does not exceed the normal evacuation time (of about 1 min) by a factor of two, insert the holder.
    3. Open the beam valve when the vacuum of the main sample chamber is in the range that allows its opening. Insert the ADF detector by pressing the ADF button.

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

* + 1. Set the microscope in continuous image acquisition mode using an image size of 512 x 512 pixels, a pixel dwell time of 2 µs, and a magnification of 20,000×. Search for the SiN window by translating the stage in the x and y directions.

Note: The microchips block any electrons passing through the sample towards the detector; signal is only visible at the location of the SiN. See Figure 5. Sometimes, it is easier to find the SiN window by viewing the fluorescence screen.

* + 1. Once the window has been found, adjust the contrast and brightness settings (using the respective knobs) so that the edge of the window becomes visible. Move the edge towards the middle of the field of view by pressing the x- and y-translation buttons of the specimen stage. Press the reset button of the objective lens.

Note: The brightness must be largely reduced compared to a vacuum sample on account of strong scattering in the liquid.

* + 1. Proceed by coarse focusing the sharp corner at the edge of the SiN window by adjusting the vertical position (z-translation) of the specimen stage. Verify that the sample position is at the eucentric high by rotating the stage by 5 degrees and rotating it back. Features of the sample and the corner of the SiN window should not shift at low magnifications.
    2. Readjust the vertical position as needed to place the window edge in the middle of the viewing area again. Store the position of the stage using the store button of the software.
    3. Move to a position where small pieces of debris or other objects of high contrast (*e.g.,* AuNPs) are present by translating the stage in the x and y directions. Adjust the focus of the objective lens so that all objects appear sharp in focus.
    4. Make note of the current density measured at the phosphor screen visible via the operating software, indicating the transmitted current through the liquid holder and through the opening of the ADF detector. Calculate the thickness of the liquid cell using Equation 1. Proceed only if the liquid thickness has been determined and does not exceed 3 µm.

Note: The thickness of the liquid cell can be determined by the ratio of the measured current density with and without the sample using the following equation14:

Equation 1

with *t*SiN the thickness of the amorphous silicon nitride membrane and *t*water the thickness of the water layer. The mean free path lengths amount to *l*SiN = 0.79 µm of SiN,= and *l*water = 3.0 µm of water for the detector opening semi-angle of 35 mrad14.

* + 1. Carefully observe the liquid at lower magnifications to ensure that gas bubbles are not present. Gas bubbles should be prevented for continuous liquid flow and the probe current.
    2. Translate the stage in the x and y directions until an area containing at least 20 AuNPs has been found. Set the image size to 1,024 x 1,024 pixels, the pixel dwell time to 19 µs, and the magnification to 400,000×. Acquire images of AuNPs adhered to the SiN membrane by pressing the acquisition button.

Note: Once images have been obtained in which the AuNPs are visible with strong contrast and sharp edges (see Figure 6), one can be sure that the experiment is correctly set up. In case unexpected problems occur, do not proceed with the experiment but make sure to resolve the cause.

* 1. **STEM of the dissolution of AuNPs**
     1. Remove the liquid flow TEM holder from the microscope and stop the liquid flow.
     2. Prepare at least 1 mL of a solution of 0.1 M of sodium chloride in HPLC-grade water in a glass syringe.
     3. Adjust the flow system to a velocity of 20 µL/min and flush the whole flow system with 0.3 mL of the saline solution. Use filter paper to collect the liquid being ejected on the other end of the tubing. Afterwards, re-adjust the flow system to 2 µL/min.
     4. Check the integrity of the SiN window using a binocular light microscope. If no leakage is observed, reinsert the liquid flow TEM holder into the microscope.
     5. Move the stage back of the microscope back to its previous position using the stored stage position. Set the microscope in continuous image acquisition mode using an image size of 512 x 512 pixels, a pixel dwell time of 2 µs, and a magnification of 20,000×. Adjust the contrast, brightness, focus, and eucentric height, as described in steps 3.1.7-3.1.9.
     6. Find a location of interest by translating the stage in the x and y directions. Set the image size to 1,024 x 1,024 pixels, the pixel dwell time to 2 µs, and the magnification to 500,000×. Record a series of STEM images by manually pressing the acquisition button once the previous image has been recorded.

Note: The AuNPs start to dissolve as soon as the electron beam is scanned over the sample. Particles outside the irradiated area are not affected and can be observed immediately after. The time stamp is stored in the image header. It is also possible to use software for the automated collection of a series of images into a movie.

* 1. **Disassembling and cleaning the TEM holder after the experiment**
     1. When the liquid-phase TEM experiments are completed and the holder is retracted from the electron microscope, clean the tubing, the liquid chamber, and its components in order to remove any solid particles or remaining salt that might influence future experiments.
     2. Loosen the rear screw slightly so that it is still fixed in its slot but the lid can be removed easily. Loosen the front screw, remove it, and place it in a dust-free environment.
     3. Remove the lid and place it in dust-free environment for storage.
     4. Remove the two microchips from the pocket. Separate them by dipping them carefully in water or in the remaining solution of the experiment. Place the microchips on tissue paper with the SiN membrane side upward and let them dry in air for further analysis.
     5. Remove the O-rings and clean the respective grooves with HPLC-grade water. Store the O-rings in a dust-free environment.
     6. Use a glass syringe (5 mL) to flush all tubing (input and output), each with 5 mL of HPLC-grade water. Collect the liquid with a small beaker placed below the TEM holder tip. After flushing, use a pipette and/or filter paper to remove the remaining liquid from the liquid compartment.
     7. Inspect the tip of the TEM holder and remove residues like broken edges of the silicon microchips, fibers, or dust. If salt is still visible, repeat the cleaning procedure. Return the O-rings to their grooves.
     8. Store the TEM holder and its components in a dust-free environment.
  2. **Procedure alternative to 2.2: STEM of moving s**

Note: A different assembly procedure is required to study the movement of AuNPs in liquid.

* + 1. Omit step 1.3. Load the AuNPs on the silicon microchip immediately before inserting them in the TEM Holder in step 2.2. Keep the sample covered by a liquid layer at all times in order to avoid a strong attachment of the AuNPs to the SiN membrane. The other steps of the protocol are the same. A series of STEM images is obtained in step 3.2.6.

*[Place Figure 4 here]*

*[Place Figure 5 here]*

**REPRESENTATIVE RESULTS:**

The liquid flow TEM holder was used to study the behavior of AuNPs in liquid. AuNPs were stably immobilized on the SiN membrane in pure water and were imaged with nanoscale resolution using liquid-phase STEM (Figure 6). Excellent contrast was obtained on the strongly-scattering gold. The current density on the phosphor screen measured for a dry test sample was 20 pA/cm², while it amounted to 8 pA/cm² with the liquid flow TEM holder inserted. Using Equation 1, *t*water = 2.4 ± 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 the AuNPs with nanometer spatial resolution. The liquid thickness was thicker than the 200 nm set by the spacer due to bulging of the SiN membranes, non-flatness of the microchips, and debris residing on the microchips.

For pure water, the AuNPs maintain their shape during imaging16, although reactive radiolysis products (e-aq, H•, H+, OH•) originating from the interaction of the electron beam with water may oxidize single gold atoms, leading to a change of shape of the AuNPs15. However, when the liquid flow system was used to introduce chloride ions in a second experiment, the stability of the AuNPs changed. Chloride ions are capable of stabilizing oxidized gold atoms in the form of tetrachloroaureat, AuCl4-. Figure 7 shows that the AuNPs slowly dissolved during a STEM imaging time-lapse series, similar to results reported earlier16. For the used electron dose rate, it took ~300 s to dissolve the 30 nm-sized AuNPs.

The movements of AuNPs in water were studied in a third experiment (Figure 8). Prior to the experiment, the liquid flow TEM holder was cleaned in order to remove any traces of salt. Differing from the first experiment, an alternative sample preparation approach was used to achieve a weaker attachment of the AuNPs to the SiN membrane14. In this experiment, the AuNP solution was placed on the silicon microchip and assembled in the liquid flow TEM holder without letting the solution dry out. In this way, the AuNPs easily detached from the SiN membrane upon imaging at the dose rate used. Some of the AuNPs moved away from the field of view into the bulk solutions, while the remaining AuNPs remained within the field of view in close proximity to the SiN window. Movements of these AuNPs were observed, and eventually they agglomerated. After a while, these agglomerates also detached from the SiN membrane and moved out of the field of view and into the solution.

**FIGURE LEGENDS:**

**Figure 1: Liquid flow cell for scanning transmission electron microscopy (STEM).** A: Schematic illustration of the assembled liquid cell. Two silicon microchips with silicon nitride (SiN) membrane windows are positioned between two O-rings. The liquid is enclosed between the SiN membrane and is thus separated from the vacuum in the electron microscope. A focused electron beam scans over the sample. Contrast is obtained from scattered electrons. Gold nanoparticles (AuNPs) are immobilized within the liquid at the SiN membrane and also move in the liquid. B: Schematic side view cross section of the stack of two microchips with O-rings.

**Figure 2: Cleaning procedure of the Si microchips.** A: Two beakers are filled with 40-60 mL of acetone and ethanol each. B: The Si microchips are placed in the beaker filled with acetone. The side with the SiN membrane should face upwards. The reflection of the two Si microchips clearly shows the groove on the backside of two microchips. C: After 2 min, the Si microchips are transferred to the second beaker filled with ethanol. After another 2 min, the Si microchips are transferred to a cleanroom tissue for drying.

**Figure 3: Liquid flow transmission electron microscopy (TEM) holder equipment.** A: The liquid flow TEM holder with plastic tubing and a syringe for liquid flow. B: The tip of the liquid flow TEM holder removed from the holder shaft, the lid of the liquid cell compartment, O-rings, and two silicon microchips. The tubing protrudes from the left side of the tip. C: The liquid cell compartment showing one O-ring, the slot for the microchip placement. D: Different tweezers on a dust-free surface (aluminum foil). E: The lid of the liquid cell compartment with its two O-rings. F: Two silicon microchips with SiN membrane windows. Left: the sample microchip without a spacer; right: the cover microchip with a 200-µm spacer. G: A microfluidic pump system.

**Figure 4: Assembling the liquid flow TEM holder.** A: The liquid cell compartment with the smaller O-ring placed in its groove. The inset shows the top view. B: The base microchip is placed in the respective socket. The inset shows the side view at such angle that the microchip is visible from light reflection. C-D: A droplet of the solution is added to the microchip. E-G: Placement of the cover microchip. H-I: Placement of the lid of the liquid cell compartment. J: Fixation of the lid with the two screws. K: Assembled liquid flow TEM holder.

**Figure 5:** **Initial positioning and focusing using STEM micrographs.** A: To locate the SiN window, the stage is moved towards the brightest signal. The silicon microchip is thin enough for some electrons to pass through close to the window. B: The edge of the focused SiN window showing some AuNPs appearing bright on the dark (no scattering) SiN membrane window. The edge of the microchip is bright due to excessive scattering. C: Focusing is done at the corner of the SiN window. The images show under-focused, in focus, and over-focused situations.

**Figure 6:** **Scanning transmission electron microscopy (STEM) micrograph of AuNPs 30 nm in diameter at the top of a pure water layer.** The image shown is a selected area of the original image. The image size was 1,024 x 1,024 pixels, the pixel dwell time was 19 µs, the pixel size was 0.73 nm, and the magnification was 400,000×. The electron dose was thus 7.1×104 e-/nm². The current density measured on the phosphor screen was 8 pA/cm2, so the liquid thickness was calculated to amount to 2.4 µm.

**Figure 7: Time-lapse series of STEM micrographs of AuNPs in saline.** A-D: Images extracted from the time-lapse series of STEM images at 30-s intervals. The AuNPs gradually dissolve in liquid as a consequence of the presence of chloride ions. The pixel dwell time was 2 µs, the frame time of the time lapse series was 1.75 s, the pixel size was 0.44 nm, and the magnification was 500,000×. The electron dose per image was 1.2×104 e-/nm². The liquid thickness was 2.4 µm.

**Figure 8: STEM micrograph of AuNPs moving in pure water.** A: SiN membrane with AuNPs, of which several are selected with arrows. B: Motion tracks of the selected AuNPs (see A). Some AuNPs move away from the field of view during the time of imaging. The remaining AuNPs move laterally along the SiN membrane and start agglomerating. Upon reaching a critical cluster size, they dispatch from the membrane and move away from the field of view. The pixel dwell time was 1 µs, the frame time was 0.52 s, the pixel size was 1.8 nm, and the magnification was 120,000×. The electron dose per image was 3.5×102 e-/nm² and the liquid thickness was 2.4 µm.

**DISCUSSION:**

The described protocol enables STEM of AuNPs in a liquid, including the observation of dynamic processes. The assembly of the holder is an easy-to-learn technique. However, several aspects must be considered when working with the liquid flow TEM holder. For instance, broken edges of the Si microchip or large particles on the O-rings may result in leakage of the liquid cell. On the other hand, large particles (>200 nm; *e.g.,* dust or Si debris) on the SiN membrane may result in an increased thickness of the liquid cell, leading to a low imaging contrast or to a low spatial resolution and may even cause SiN windows to break. Importantly, residues of salt or other chemicals may influence the outcome of the experiments in an undesired way. Therefore, it is crucial that the different steps of sample preparation and holder assembly are carried out carefully and in a clean and dust-free environment.

The thickness of the liquid cell determines the achievable resolution, as well as the contrast of the obtained images17. This thickness can be adjusted via spacers located on one of the two Si microchips. Depending on the dimensions of the sample, different thicknesses of the liquid cell can be realized. For the study of AuNPs, it is possible to use small spacers (200-500 nm), while whole eukaryotic cells need larger spacers of up to 5 µm. The thickness of the liquid cell is further influenced by the bulging of the SiN membrane windows resulting from the pressure difference between the liquid cell and the surrounding vacuum. This effect becomes more pronounced with larger SiN membrane windows. Thus, in order to minimize the thickness of the liquid cell, it is recommended to use small SiN membrane windows. In case it is difficult to find an overlap between two small windows, they can be assembled in a crossed configuration using a different base microchip. Alternative configurations largely prevent bulging and consist of a monolithic microchip18 or membrane windows supported by pillars19, but those exhibit disadvantages regarding sample loading. One of the most challenging aspects of the current technology is the lack of precise control over the liquid thickness. Often, the liquid is much thicker than what is expected from the spacer dimensions used, as was shown here. Several groups used closed liquid chambers4,20-22; these systems have some advantages regarding spatial resolution, as the liquid thickness can be reduced by inducing a bubble in the liquid. Alternatively, the SiN windows can be forced to collapse, leading to a thinner liquid layer. Thirdly, the enclosure of other thinner windows exists (*e.g.,* graphene)23, also resulting in much thinner liquids than what is possible with the system described in this protocol. However, it is impossible to flow liquid in those systems.

As for any high-resolution microscopy technique, a number of experimental aspects must be considered. The most important aspect is the interaction of the electron beam with the liquid or the sample. In addition to radiation damage, which limits the achievable spatial resolution for many solid samples24, the liquid samples are also influenced by electron beam-generated radiolysis products15,25. Since these products may influence the experiment, careful data interpretation and experimental design are essential26. The microscope settings should be chosen according to the objectives of a particular study. ADF STEM is more powerful for imaging nanoparticles of a high atomic number (Z) in larger thicknesses of the liquid cell, while TEM gives better contrast on low-Z materials and is typically faster but requires thinner liquid layers3. Instead of using the ADF detector, the bright field (BF) detector is sometimes used to image the liquid cell, since BF STEM is advantageous for imaging low-Z materials in thick layers27. With increasing thickness of the liquid cell, more current is needed. However, this also increases the concentrations of radiolysis products and increases radiation damage. It should also be noted that an inversion of contrast is observed in the ADF detector for very thick liquids (>10 µm for water).

The liquid conditions were changed between our experiments by removing the holder from the microscope and exchanging both the sample and the liquid. In addition to changing the salt concentration, it is readily possible to change other properties of the liquid by flowing in different liquids (*e.g.,* one may use buffer solutions in order to set a specific pH16 or may introduce organic solutions or other additives). It is also possible to change the liquid while the holder is still inserted in the microscope by flowing liquids through the microfluidic system. However, in this case, it is unknown at which time point the liquid at the sample changes. It is also noteworthy that microchips supporting electrodes are available, so nanoscale electrochemistry experiments can be carried out28.

The objects of study are not limited to AuNPs in water, but a wide variety of specimens can be studied using the protocol described above, including silica, titanium oxide, and polymers. If movements of the objects are too fast to capture in an image within the acquisition, the viscosity can be reduced by an order of magnitude by using a mixture of 50% glycerol and 50% water.

From the aforementioned points, a number of advantages, possibilities, and also disadvantages become apparent. When working with liquid-phase STEM, the most important disadvantages to consider are that: 1) any experiment is influenced by the dynamic interaction of the electron beam with the entire specimen (the object under observation, the liquid, and the SiN membranes); 2) sample handling is tedious, and it is often difficult to achieve a thin liquid layer because the sample or the microchips contain some micrometer-sized particles; 3) the liquid thickness usually differs largely from the intended thickness set by the spacer; and 4) spatial resolution and contrast strongly depend upon the liquid thickness and the difference between the change density of the object under observation and the liquid.

Presently, ample methods exist for the microscopy of objects in liquid with nanometer spatial resolution. Electron microscopy in amorphous ice is a powerful technique29, but the involved experimental procedures are delicate, not all experiments allow the preparation of the sample in ice, and time-resolved experiments are impossible. X-ray microscopy30,31 could in principle be used, but it has a limited spatial resolution and is not widely available in laboratories. Atomic force microscopy in liquid has been established but is a surface technique only32-35. Light microscopy does not exhibit sufficient spatial resolution. At the present, electron microscopy in liquid seems the most powerful technique for direct microscopy of nanoscale objects and processes in liquid.

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.

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**DISCLOSURES:**

The authors have nothing to disclose.

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