Published in final edited form as:

J Micromech Microeng. 2014 November 1; 24(11): 115001–. doi:10.1088/0960-1317/24/11/115001.

Gas-Assisted Annular Microsprayer for Sample Preparation for Time-Resolved Cryo-Electron Microscopy

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Abstract

Time-resolved cryo electron microscopy (TRCEM) has emerged as a powerful technique for transient structural characterization of isolated biomacromolecular complexes in their native state within the time scale of seconds to milliseconds. For TRCEM sample preparation, microfluidic device [9] has been demonstrated to be a promising approach to facilitate TRCEM biological sample preparation. It is capable of achieving rapidly aqueous sample mixing, controlled reaction incubation, and sample deposition on electron microscopy (EM) grids for rapid freezing. One of the critical challenges is to transfer samples to cryo-EM grids from the microfluidic device. By using microspraying method, the generated droplet size needs to be controlled to facilitate the thin ice film formation on the grid surface for efficient data collection, while not too thin to be dried out before freezing, i.e., optimized mean droplet size needs to be achieved. In this work, we developed a novel monolithic three dimensional (3D) annular gas-assisted microfluidic sprayer using 3D MEMS (MicroElectroMechanical System) fabrication techniques. The microsprayer demonstrated dense and consistent microsprays with average droplet size between 6-9 μm , which fulfilled the above droplet size requirement for TRCEM sample preparation. With droplet density of around 12-18 per grid window (window size is 58 $\times$ 58 μm), and the data collectible thin ice region of >50% total wetted area, we collected ~800-1000 high quality CCD micrographs in a 6-8 hour period of continuous effort. This level of output is comparable to what were routinely achieved using cryo-grids prepared by conventional blotting and manual data collection. In this case, weeks of data collection process with the previous device [9] has shortened to a day or two. And hundreds of microliter of valuable sample consumption can be reduced to only a small fraction.

Keywords

Microfluidics; micronozzle; microspray; microdroplet; monolithic device; cryo EM; time-resolved TEM

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Introduction

As is well appreciated by structural biologists, knowledge of a single static molecular structure of a complex macromolecular assembly (e.g. a ribosome or a virus) is insufficient to understand the mechanisms by which the complex performs its functions. In some cases, biochemical modification allows for the elucidation of structures for complexes that are trapped in distinct functional states; however, application of this approach is limited. Ideally, ‘snapshot’ structural models of the complex in all the conformational states that are relevant to its functioning are desirable, but existing biophysical techniques capable of providing such detailed information are inadequate [1-3]. One promising biophysical technique in this area is cryo-electron microscopy (cryo-EM) of frozen-hydrated, isolated macromolecular assemblies in conjunction with single-particle image processing [4]. Transient structural states of macromolecular complexes can be viewed with cryo electron microscopy by rapid quenching to liquid nitrogen temperatures. However, the potential for time-resolved cryo-electron microscopy (TRCEM) to provide structural details about the dynamics of macromolecular assemblies remains largely unrealized due to technical limitations and difficulties in applying existing technologies [5-8]. The key challenge for time-resolved cryo-EM remains how to rapidly mix reactants, incubate them for a specific time (usually from a few milliseconds to seconds), and deposit the products in a thin aqueous film (less than 200 nm in thickness) on electron microscope grids for near-instantaneous freezing.

In the past works [9-10], we have made major strides in the development of microfluidic devices for performing TRCEM on reacting solutions of macromolecular complexes. The devices integrated an ultrafast micromixer, and a two dimensional (2D) in-plane gas assisted liquid microsyringer in a monolithic microchip. An important feature of these devices is that the bulk of the reaction does not take place while the specimen is adhering to the carbon support film of the EM grid, as is the case in other techniques, but, it rather takes place in solution prior to deposition on the grid. The devices are capable of fully mixing two reactant solutions, spraying the mixed solution on an EM grid and freezing it in times as short as 10 milliseconds. Using the device we have observed reassociation of 30S and 50S ribosomal subunits to form 70S ribosomes. This technology will allow TRCEM to reach its potential to produce the structural “snapshots” necessary to study the mechanisms by which bio-macromolecular complexes perform their functions.

In addition to the requirement of millisecond time resolution, there are other challenges that one has to overcome to make TRCEM an efficient technique which can be routinely applied in any laboratory that is proficient at cryo-electron microscopy. One critical challenge is the microspray droplets size control to improve the data collection efficiency and reduce the consumption of valuable samples. Microdroplet generation is one of the major microfluidic research area [11], the applications including ink-jet printing [12], biomedicine [13], chemical analysis [14], fuel-injection [15], etc. Numerous operating mechanisms and designs have been developed [11-14, 16-18], including piezoelectric droplet ejection, acoustic-wave actuation, thermal, and electrostatic actuation. These methods has the advantages of generating micro-sized droplets with accurately controlled droplet size and number. However, the system complexity and the droplet throughput are the major obstacles

to apply them in our application, which requires minimum reactant residence time dispersion and the least time of droplet-grid interaction. Furthermore, these techniques commonly involve high electrical or acoustic energy to generate and disperse microdroplets, which can impose detrimental effects on potentially fragile macromolecular complexes and their activities. On the other hand, the gas-assisted spray nozzles, which utilize the interaction of gas and liquid flows to generate microdroplets, has the advantage of fast atomization speed, low device cost, and flexible system integration capability [17, 19-20]. With MEMS fabrications techniques, nozzles with dimensions of tens of microns, or even scaled down to several microns can be fabricated, and the droplet size can be reduced down to several microns with the sample consumption reduced to sub-microliters per minute.

In this study, the droplet size generated by the micronozzle needs to be designed to facilitate the thin enough ice film generation on the grid surface, while not too thin to be dried out before freezing. That means an optimized mean droplet size ($\sim 5\text{-}10\text{ }\mu\text{m}$) needs to be achieved. Normally, for cryo EM sample grid preparation, the grid is coated with a thin layer of continuous carbon film and glow discharged to render the surface hydrophilic. This process facilitates droplet spreading and improves thin ice formation on the grid surface. Smaller droplets with less liquid volume tend to spread easier after contacting the grid surface, and form more thin ice region than larger ones before freezing in a very short time (usually in milliseconds). In the previous work, 2D in-plane nozzle generated sparse and large droplets (with mean droplet size larger than $\sim 20\text{ }\mu\text{m}$). The large droplets were found to be difficult to form sufficient thin ice regions due to spreading difficulty in a limited time (in milliseconds) before freezing. Thus only the edge of the large droplets ($<15\%$ in total wetting regions) was thin enough for data collection. Thus ice regions with contrast suitable for high resolution imaging are highly limited [9]. The consequential data collection process was considerably inefficient. It consumed large sample quantity due to the need for preparing many EM grids, and thus large scale dataset collecting became difficult.

In this work, we focused on design and develop a novel type of three dimensional (3D) annual gas-assisted micronozzle sprayer. The new design was implemented using 3D MEMS (MicroElectroMechanical System) microfabrication techniques and tested with the goal of further reducing the mean droplet size and increasing the droplet density to form sufficient thin ice regions for data collection.

Device Design

The previous 2D microspray nozzle [10] consisted of in-plane shallow microfluidic channels with rectangular cross-sections, two of which carried gas and sandwiched a third channel that transported the aqueous reaction mixture. This type of in-plane nozzle structure induces significant gas resistance inside the gas channel, and limits the mass flow rate of the gas available for liquid atomization. Theoretically, the droplet size and its size distribution generated by a nozzle largely depend on the gas-liquid volumetric flow rate ratio, as indicated by the following theory for a prefilming air/gas assist/blast atomizer (in which liquid is pre-formed as a thin film and is discharged as a thin sheet at the nozzle opening) [21]. The Sauter Mean Droplet Size (SMD) of the nozzle is determined by the following equation [21]:

$$\frac{SMD}{L_c} = A \left(\frac{\delta}{\rho_A U_A^2 D_p} \right)^{0.5} \left(1 + \frac{\dot{m}_L}{\dot{m}_A} \right) + B \left(\frac{\mu_L^2}{\delta \rho_L D_p} \right)^{0.5} \left(1 + \frac{\dot{m}_L}{\dot{m}_A} \right), \quad (1)$$

where L_c is the characteristic dimension of the atomizer, and A and B are constants related to atomizer design and can be determined experimentally. δ is the liquid surface tension, ρ_A and ρ_L are the gas and liquid density, $\dot{m}_A$ and $\dot{m}_L$ are the mass flow rates of the gas and liquid, U_A is the gas velocity at the nozzle exit, μ_L is the liquid dynamic viscosity, and D_p is the prefilmer nozzle diameter.

In general, the mean droplet size is proportional to liquid viscosity and surface tension, and inversely proportional to gas pressure, the relative velocities of gas and liquid, and mass flow rate ratio of gas and liquid. The droplet size is determined by the atomizer geometry as well, such as liquid and gas contact region.

Based on the above theory, the novel nozzle design included a 3D gas-liquid-gas annular nozzle structure to increase liquid-gas contact region, as shown in Figure 1. The gas flow is introduced from the back side of the device, and then blow out vertically to the front surface through the gas nozzles. The in-plane liquid flow is first parallel to the device surface, and then changes to vertical direction and reaches the device surface as a thin film between the annular gas nozzle openings. The liquid and gas meet in parallel at the surface where atomization occurs. The thin liquid films are surrounded by the gas flows. The design significantly increases the liquid-gas contact region, and facilitates the breakdown of the liquid films into small droplets. The nozzle design minimizes the resistance of the gas channels and would easily control the mass gas flow rate available to the liquid atomization, thereby helps to reduce the droplet size. Another potential advantage is that, the distance between the micromixer and the micronozzle can be significantly reduced as comparing to the 2D in-plane nozzle design, thus further reduces the incubation time for the reaction mixture. It has the potential to extend the time scale to sub-millisecond region for time-resolved EM study [9].

The specific scheme and dimensions of the circular nozzle designs used in this study are shown in Figure 2. The overall dimensions of the nozzle range from several hundred micrometers to slightly more than 1.0 millimeter. The annular-shaped outer gas nozzle openings have a width of 100-200 μm , the inner gas round-shaped nozzle opening have a diameter of 200 μm , and the two small arc-shaped liquid nozzle openings have a width of 50 μm . The geometrical parameters selected are the considerations of both the previous device design parameters and the 3D MEMS microfabrication technical difficulty and limitation.

Device Fabrication

The device fabrication procedure is illustrated in Figure 3. In addition to the annular nozzle patterns, the photomasks contained micromixer patterns as well. The micromixer has been elaborated in the previous work [10]. The purpose of the mixer integration is for future studies on potential biological reactions. As shown in Figure 3, A 4-inch-diameter silicon wafer (Wafer 1) of 325 μm thickness and coated with a thin layer of 2.0 μm silicon oxide on

one side. The other side was spin-coated with a 20.0- μm thick SU-8 photoresist. Then, the micronozzle patterns were transferred from the mask to the SU-8 resist layer and DRIE etched in Alcatel AMS100 DRIE etcher (Alcatel Vacuum Products, Tempe, AZ) to produce the front liquid/gas nozzle openings. Another 4-inch-diameter silicon wafer (Wafer 2) of 525 μm thickness was spin-coated on the front side with a 1.0- μm thick positive 1813 photoresist. The silicon wafer was DRIE etched to produce 40- μm deep liquid microchannels. Then, the gas/liquid inlet patterns were aligned and transferred to the back side coated with a 20.0- μm negative SU-8 photoresist, and DRIE-etched through the whole wafer to produce the backside liquid/gas inlets of the device. The two wafers were diced into individual chips using a ThermoCarbon Tcar 864-1 dicing saw (Thermocarbon Inc., Casselberry, FL). The corresponding chip pair were then aligned and bonded to each other so as to seal the microchannels and finalize the device.

The bonding was conducted using a thin layer of 1.0 μm Polyset epoxy siloxane (PES) polymer [22]. The bonding process was carried out at 165°C for 1.0 hour using a Fineplacer Pico Ma multipurpose die bonder (Finetech USA, Tempe, AZ) with alignment. After the bonding process, the device was immersed in BOE buffer with sonication for 30 min to remove oxide covering the micronozzle. As the final step, three Nanoport assemblies (Upchurch Scientific, Oak Harbor, WA) were attached to the device as fittings for tubing connections. The process resulted in strong PES bonding of the two silicon chips: No liquid leaking was observed with the flow rate up to 10 $\mu\text{L/s}$. SEM micrographs of one typical pair of fabricated micronozzles before bonding are shown in Figure 2.

Prior to each experiment, the device was cleaned by flushing microchannels with 4.0 mL double-distilled, deionized, autoclaved water, followed by 2.0 mL of buffer solution [9].

Device Evaluation Methods

Microspraying evaluation

The performance of the device in generating microdroplets was first evaluated by spraying deionized water onto carbon-coated hydrophobic EM grids. The grids were then examined using a cryo EM. A ~20 nm hydrophobic carbon thin film was pre-coated on each 3-mm diameter holey TEM grid before the testing. The carbon film was deposited to reinforce the original holey carbon films on the grid. Then a thin (5 nm) continuous carbon support film was transferred from a mica substrate to the grids to coat the holes on the grids. The carbon film was coated to the grid using a Bal-Tec MED 020 high vacuum coater (Leica Microsystems) without further treatment. The grid surface was deliberately kept hydrophobic in the tests to minimize the droplet spreading on the grid surface, in order to evaluate the droplets size.

The testing system setup scheme is shown in Figure 4. The device was held vertical in the holder with the nozzle facing the EM grid plunger. Deionized water was fed to the device from the back side through 1/16" polyetheretherketone (PEEK) capillary tubing (125 μm inner diameter) and 2.0- μm PEEK micro filters by a dual syringe pump (Cole Parmer Inc. Vernon Hills, IL). The flow rate was maintained constant at 6.0 $\mu\text{L/s}$ for all the experiments. The liquid flow rate was pre-determined and restricted by the upstream

micromixer to assure complete liquid mixing for future biological reactions [10]. Nitrogen gas was fed into the gas nozzle by a nitrogen tank from the back side as well. The regulated pressures at the gas tank were 20 and 40 psi.

Each grid to be tested is held on a sharp-tip tweezers, and was aligned and plunged through the spray into liquid ethane cryogen. The plunge speed (1.0 m/s) was controlled by a computer-controlled grid freezing instrument [23]. The grid containing the collected microdroplets was instantaneously frozen in the liquid ethane, and placed in a TEM grid holder. The sample was then examined using an FEI Tecnai F20 transmission electron microscope (FEI Company, Hillsboro OR) for droplet size evaluation.

Ice Thickness and EM Data Collection Evaluation

On the contrary to the droplet size evaluation experiments, for cryo EM imaging of actual biological samples, it is important to render the EM grid surface hydrophilic in order to induce the droplet spreading for thin ice formation. Although the ice thickness can be estimated from micrographs by comparing densities of ice-containing and ice-free regions of the grid, the more efficient and convenient method is to evaluate the ice thickness from the image contrast (signal to noise ratio) of the biological samples (such as 70S *E. coli* ribosome particles): when the contrast of the ribosome is good, the ice must be less than 1,000 angstrom in thickness. In this study, we used the latter method to do ice thickness evaluation.

In the test, prior to using the grids, the carbon coated grids were glow discharged for 30 seconds using a plasma cleaner (medium setting on Harrick model PKC-3xG) to render surface hydrophilic [25]. To evaluate the ice thickness and the available data collection region from the generated microdroplets, we sprayed the 70S *E. coli* ribosomes suspensions (final concentrations 2.0 μM) at room temperature onto the cryo-EM grids and examined the microdroplet spreading and thin ice forming using TEM. Ribosomes were purified as described in the literature [24] with a slight modification. Ribosome samples were prepared in polymix buffer (95mM KCl, 8mM putrescine, 5mM KH_2PO_4 , 5mM NH_4Cl , 5mM magnesium acetate, 1mM spermidine, 1mM dithiothreitol, 0.5mM CaCl_2 , (pH 7.3)). The selected microsyringe was mounted onto the computer-controlled grid-plunging device. Ribosomes were then loaded in one syringe, sprayed (with flow rate 6 $\mu\text{L/s}$) onto R2/4 Quantifoil grids (Micro Tools GmbH, Jena, Germany), and plunged into liquid ethane for freezing.

EM evaluation was performed on the F20 platform operating at 200 kV. The TEM was configured at nominal 50,000X magnification (50,360X calibrated), under low-dose conditions, and with defocus ranging from 2.3 to 4.4 μm under focus. Micrographs were collected using a 4K CCD camera (*TIETZ F415MP* 4k x 4k multiport CCD camera (4096 $\times$ 4096 pixels). Number of ribosome micrographs collected from the spread droplets in which ice thickness was equivalent to that produced by the conventional blotting methods (~ 100 nm or less) was evaluated.

Device Test Results and Discussion

Micronozzle Spraying Characterization

Three fabricated devices (differing from one another in the geometrical dimensions of the liquid and gas nozzle openings) specified in Table 1 were tested in this study. Generally, Device #1, #2, and #3 have the overall nozzle dimensions of 1100, 700, and 900 μm in diameter, respectively. The gas pressures were controlled constant at 20 & 40 psi using a pressure regulator at a nitrogen tank. The nitrogen gas passed through two GE bubbling humidifying filters via a 2-foot long, 1/8 inch silicon tubing to saturate the humidity and then fed to the device via 12 cm long capillary tubing with the inner diameter of 1.2 mm. The gas volumetric flow rate was measured using a flow meter with maximum scale of 10.0 SCFH.

As shown in Figure 4(c) as an example, all the devices generated dense and steady sprays at the testing gas pressures. With regulated gas pressure of 20 and 40 psi, the gas flow rates were in the range of 4.0 to 7.0 standard cubic feet per hour (SCFH). The actual spray angle was wider than the laser illuminated portion shown in the photo: At about 2.0 cm away from the nozzle, the micro spray size reached 0.5-1.0 cm in diameter. The sprays were strong enough to reach beyond 2 feet from the nozzle while still remained steady. This is different from the in-plane nozzles tested in the previous study [9], which was much weaker and less steady; with gas mass flow rate was less than 1.0 SCFH at a regulated gas pressure of 50 psi. Figure 5(a) showed a typical cryo-EM micrograph of deionized water microdroplets sprayed on a quantifoil grid window. As shown in Table 1, for all the spray conditions tested, the average droplet diameter calculated is between 6 to 9 μm , and the droplet density per grid window is around 12-18. Note that the microdroplets with diameter less than 2 μm were neglected because they were difficult to track and to collect data due to the tiny size. The results showed a significant spraying improvement comparing to the in-plane nozzles [9]. The in-plane nozzle showed sparse and much larger droplets with a diameter of mostly larger than 20 μm , with the droplet density of 2-3 per window.

As shown in Figure 6 as well, most of the droplet size fell in the range of 2-10 μm . Another interesting aspect is that, for all the three devices tested, as shown in Table 1 and in Figure 6, their droplet size distribution were very similar, and no significant difference among nozzles with different dimensions can be found. The results indicated that the device spray performance is mainly determined by the nozzle geometrical configurations. The results were evidently different from that of the in-plane nozzle configuration which showed average droplet size of $\sim 20 \mu\text{m}$ and even larger [9]. It demonstrated that the current design significantly reduced the gas resistance in the gas flow path, increased the gas volumetric flow rate and extended liquid-gas contact region for liquid atomization, thus generated much smaller droplets.

As to the nozzle performance with different gas pressure, we found that, for all the devices, the average droplet size generated at 20 psi is slightly larger than that at 40 psi. On the other hand, at 40 psi, all the devices generated more droplets than that at lower pressure. The results were shown in Table 1 as well. Another aspect worthy of discussion is that, because the spray from the present devices was much stronger than the device with the 2D in-plane

nozzle configuration, the perforated carbon film of the Quantifoil holly grids needed to be reinforced with an additional carbon coating (20-30 nm) before application of the thin (5 nm), continuous carbon support film. The additional carbon film was applied to compensate the damage from the microdroplet impact. The usual carbon films with thickness of several nanometers were first tested at 20 psi. But the results showed that more than 90% of the carbon film was damaged in the experiments. After the carbon film thickness was increased to ~20 nm, the film damage was reduced significantly, as shown in Table 1. For gas pressure at 40 psi, the film damage area was around 40-50% for all the devices tested, except for device #1, which had larger than 60% damage due to the large gas nozzle openings. At gas pressure of 20 psi, the carbon film damage for the device #2 and #3 was 20-30%. We also tested the devices at gas pressure of ~10 psi (data not shown). The results indicated that less carbon film damage occurred. However, many more large droplets (>10 μm) were generated. In the future biological studies we will select the gas pressure at 20 psi for all device tests and sample preparations.

Ice Thickness evaluation and EM Data collection

Figure 5(b) showed a typical cryo-EM micrograph with frozen ribosome particles. The micrograph was obtained from small droplets dispersed on the hydrophilic EM grid. The ribosome particles in the micrograph appeared to be in good integrity and intact through the spraying process. A few of ribosome particles are highlighted in the micrograph. The 70S *E. coli* ribosomes are compact asymmetric macromolecular complexes that are about 23 nm in diameter. The particles presented a variety of appearances because they lied in the grid in multiple orientations. The appearances of the ribosomes in the micrograph are typical of those obtained by conventional cryo EM [25]. Generally, due to the smaller size of the droplets, more thin ice regions were formed during sample preparation step and were available for data collection. The micrographs with high quality (high signal-to-noise ratio) are significantly more prevalent than the 2D in-plane nozzles. As for the output of the data collected from the EM grids, we collected ~800-1000 high quality CCD micrographs (1024 x 1024 pixels) in a 6-8 hour period of continuous effort, and we estimate that an equivalent amount of data could have been obtained with additional effort. This level of output is comparable to what were routinely achieved using cryo-grids prepared by conventional blotting and manual data collection; and many more micrographs were collected using the new device than in the previous study. As a point of reference, previously only 200-300 CCD micrographs could be collected from one TEM grid that were of acceptable signal-to-noise ratio, and weeks of EM works are required to collect a reasonable size of data set for analysis.

Conclusion

In this study, we have demonstrated a novel 3D annular microspray nozzle design for time-resolved cryo-EM sample preparation. The devices were fabricated using 3D MEMS fabrication techniques. The experimental results showed a significant improvement in multiple aspects over the previous 2D in-plane nozzle design. The microsprayer demonstrated dense and consistent sprays with average droplet size between 6-9 μm . With droplet density of around 12-18 per grid window and the EM data collectible thin ice region

of >50% total wetted area, ~800-1000 high quality CCD micrographs were collected in a 6-8 hour period of continuous effort. This level of output is comparable to what were routinely achieved using cryo-grids prepared by traditional blotting and manual data collection. The appearances of the ribosomes in the micrograph are typical of those obtained by conventional cryo EM. The results indicated that the new annular nozzle design would significantly increase the data collection efficiency and output, and reduce the number of the prepared sample grids for a single experiment, thus considerably reducing the consumption of the sparse and valuable biological samples.

Acknowledgements

This work is supported by the Wadsworth Center's Resource for Visualization of Biological Complexity (NIH Biotechnology Resource Grant RR01219). We thank Dr. Pei-I Wang at Rensselaer Polytechnic Institute for his help on the PES bonding process. We acknowledge the Micro and Nano Fabrication Clean Room at Rensselaer Polytechnic Institute for their help on device fabrication.

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Figure 1.
A schematic cross-section view of the new gas-assisted microsprayer design.

Figure 2. Nozzle design schemes (top), and SEM micrographs (Bottom) of fabricated gas/liquid nozzles and liquid channels on two silicon chips

Note: The edge artifacts on wafer 2 nozzle are small pieces of thin Si debris left during the back side DRIE etching through the wafer. They could be removed by sonication during device cleaning.

Figure 3.
The fabrication procedure for the designed annular nozzles.

Figure 4. Experimental Setup with photograph of sprays generated by one of the devices
 Top diagram is the experimental setup; Bottom photographs are the actual experimental system. (a) Side view of the device mounted on the device holder; (b) front view of the device; and (c) microspray generated which was illuminated by a green-color laser beam. The annotations are (1) device itself; (2) micronozzle; (3) liquid feeding connections; (4) gas feeding connections, right on the back of the nozzle; (5) cryo-EM grid mounted on the grid plunger, (6) cryogen (liquid ethane) holder, (7) device holder, and (8) microspray generated at a gas pressure of 20 psi.

**Water droplets sprayed on EM grid
(regulated gas pressure 20psi)**

**Cryo-EM image of 70S ribosome
particles**

Figure 5. Cryo EM micrograph of droplets (a) and a 70S ribosome micrographs (b)
The results were obtained from device #3. The cryo EM grid window dimension showed in (a) is $58 \times 58 \mu\text{m}$.

Figure 6. Droplet size distributions for the three tested nozzles listed in Table 1

Note: the droplet counts were calculated by using the droplets at certain size range as a fraction (percentage) to the total droplets number counted on the grid.

Table 1

Nozzle dimensions and spraying characterization

Device #	Diameter of inner gas nozzle opening	Width of liquid nozzle opening	Width of outer gas nozzle opening	Width of baffle between nozzle opening	Gas Pressure Psi (Flow rate: SCFH)	EM Grid Carbon Coating Damage [*]	Average Droplet Diameter μm	Average Droplet Density/ grid window
1	200 μm	50 μm	200 μm	100 μm	20 (5.2)	~40-50%	7	14
					40 (6.8)	>60%	6	14
2	200 μm	50 μm	100 μm	50 μm	20 (4.4)	~20-30%	9	14
					40 (5.8)	~40%	7	18
3	200 μm	50 μm	100 μm	100 μm	20 (5.2)	~20-30%	8	12
					40 (6.5)	~50%	6	15

^{*} For EM grid carbon coating damage, the damage was calculated by using the ratio (percentage) of the broken carbon film area to the total area of carbon film on the grid.