Investigating hepatitis C virus infection using super-resolution microscopy

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Abstract

Super-resolution microscopy (SRM) can provide a window on the nanoscale events of virus replication. Here we describe a protocol for imaging hepatitis C virus infected cells using localization SRM. We provide details on sample preparation, immunostaining, data collection and super-resolution image reconstruction. We have made all efforts to generalize the protocol to make it accessible to all budding super-resolution microscopists.

Keywords

Microscopy, super-resolution, imaging, virus, hepatitis.

1 Introduction

Since the mid-1940s visualisation of virus particles and infected cells by electron microscopy (EM) has been a mainstay of virology. Whilst EM

provides an unrivalled resolution it also has severe limitations, such as the inability to observe living samples and difficulties in identifying specific molecular components. In contrast, fluorescence microscopy is minimally invasive, making it ideal for live imaging, and offers a variety of means for specific molecular labelling (e.g. antibodies or fluorescent protein tags). Nevertheless, the intrinsic diffraction limit of conventional fluorescence microscopy prevents structures smaller than ~300 nm from being resolved, therefore limiting its use for studying the molecular events of virus replication.

However, recent advances in optical physics, data analysis and photochemistry have brought about a new era of super-resolution microscopy. SRM is able to overcome the diffraction limit of conventional fluorescence microscopy to provide ultrastructural information of virus particles and infected cells that was previously only accessible by EM. As such, SRM is likely to supersede EM to become an essential component of the virologist's tool kit [1]. The various SRM techniques differ wildly in their technical and analytical approaches, the details of which can be found in these review articles [2, 3]. In this chapter, we outline the use of one SRM technique, localization microscopy, to image hepatitis C virus (HCV) infected cells.

In conventional fluorescence microscopy, a sample is decorated with a specific label (e.g. a fluorescent dye), the individual molecules of which are usually extremely crowded; consider the many thousands copies of HCV proteins that associate with intracellular membranes in infected cells. When illuminated, these fluorescent molecules emit light simultaneously and, due to

the intrinsic diffraction of light, their signals spread and merge such that the fine detail of their molecular distribution is lost. This limits the resolution of conventional fluorescence microscopy to 200-350nm.

In localization SRM techniques, such as STORM and PALM [4, 5], photochemical tricks are used to induce fluorescent dyes and proteins to photoswitch, such that they stochastically turn on and off (commonly referred to as ‘blinking’). Under these conditions, closely apposed molecules are unlikely to emit light simultaneously and the signals from individual fluorescent molecules can be captured in isolation. Mathematical fitting of these individual signals allows the true location of fluorescent molecules to be estimated with a high precision. Prolonged imaging allows thousands or millions of fluorescent molecules to be located and mapped, resulting in a high-resolution reconstruction of the molecular distribution of fluorescent label across a sample (**Figure 1)**.

Localization SRM does not require a particularly specialised (or expensive) microscope [6] and there is a wealth of open-source analysis software packages available for image reconstruction [7], making it accessible even to novice microscopists. Here we provide a detailed description of SRM of HCV infected cells, paying particular attention to the measures that need to be taken to ensure optimal sample preparation. We provide a step-by-step guide to acquiring high-quality data and performing image reconstruction using a state-of-the-art SRM analysis package [8]. The protocol outlined here uses

immunostaining of fixed samples, however, it could be adapted for live-cell SRM of fluorescently tagged virus proteins and/or virions.

2 Materials

1. #1.5 25 mm circular coverslips.
2. Sterile double deionized H_20.
3. Absolute ethanol.
4. Absolute methanol.
5. Acetone.
6. 3-Aminopropyltriethoxysilane (APES) (Sigma Aldrich).
7. Wash-N-Dry coverslip holder (Sigma Aldrich).
8. Fine point tweezers.
9. 19G syringe needle.
10. 6 well culture plate.
11. Cell media: DMEM, supplemented with 10% foetal calf serum (FCS), non-essential amino acids, penicillin and streptomycin.
12. Infection media: DMEM, supplemented with 3% FCS, non-essential amino acids, penicillin and streptomycin.
13. Cells: Huh-7.5 cells (Apath, LLC).
14. Virus: J6/JFH HCVcc stock [9]
15. Phosphate Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4.
16. Blocking buffer: PBS + 1% bovine serum albumin (BSA).
17. Antibody buffer: PBS + 0.5% BSA.

18. Primary antibody: 9E10 anti-NS5A (Charles M. Rice, Rockefeller University, New York).
19. Secondary antibody: anti-mouse IgG Alexa Flour 647 (ThermoFisher Scientific).
20. DAPI (4',6-Diamidino-2- Phenylindole, Dihydrochloride).
21. Post-fixing buffer: PBS + 1% EM grade formaldehyde.
22. Attofluor chamber (ThermoFisher Scientific).
23. Imaging buffer: PBS + 3% OxyFluor (Oxyrase Inc.), 100 μM DL-lactate, 50 mM β-Mercaptoethylamine hydrochloride (MEA), pH adjusted to 8.0-8.5 with NaOH.
24. This procedure can be performed on many intermediate or advanced imaging systems. In this case the work was performed on a Zeiss Elyra PS.1 fitted with a 100 X TIRF objective and an EMCCD camera (iXon Ultra 897, Andor). Imaging was performed using 405 nm (50 mW) and 642 nm (150 mW) diode solid-state lasers, with Band Pass 420-480/ Long Pass 655 and Long Pass 655 emission filters.
25. ImageJ/FIJI open source image analysis software with the NanoJ Super-Resolution Radial Fluctuations (SRRF) plugin installed (see section 3.6) [8, 10, 11].

3 Methods

3.1 Cleaning coverslips

SRM is particularly susceptible to background sources of fluorescence; therefore, coverslip cleaning is essential for optimal imaging. In our

experience it is preferable to prepare fresh coverslips on the day of each experiment.

1. Place a #1.5 25mm coverslip in to each well of a 6 well plate for cleaning.
2. Perform two 5 minute washes in 2 ml ddH_2O, agitate gently (**Note 1**).
3. Perform two 5 minute washes in 2 ml 100% ethanol, agitate gently.
4. Perform two 5 minute washes in 2 ml 100% methanol, agitate gently (**Note 2**).
5. Replace methanol with 2 ml ddH_2O.

3.2 Coating coverslips

Cleaned glass coverslips are typically coated with a suitable substrate to promote cell adhesion. Treatment with APES covalently coats the glass with a layer of positively charged free amine groups; this provides superior adhesion and can also be used to immobilize virus particles or purified proteins.

1. In a fume hood, prepare two 100 ml glass beakers, one containing 80 ml 100% acetone, the other 80 ml 2% APES diluted in acetone.
2. Carefully transfer the cleaned coverslips in to a Wash-N-Dry coverslip rack (**Note 3, 4**).
3. Using tweezers, dunk the coverslip rack in to the beaker of acetone, then transfer to the beaker containing 2% APES, ensuring complete immersion of the coverslips, incubate for 2 min.

4. Transfer the coverslip rack back in to the beaker of acetone to wash off excess APES. Carry the beaker to a biosafety cabinet, retrieve the coverslip rack and set aside to air dry for 15 min. Discard the acetone and APES (**Note 5**).

3.3 Sample preparation

The precise nature of the sample(s) will depend on the particular experiment; nonetheless, careful sample preparation is critical to successful SRM. This simple protocol generates HCV-infected cell samples for imaging, without any particular experimental question in mind. SRM is a powerful but time consuming approach, we would recommend using it to address focused questions, with a small number of experimental parameters. More extensive studies with multiple parameters and/or treatments are better suited to lower resolution imaging techniques; SRM should be used to provide fine details.

1. Once dried, place one prepared coverslip into each well of a 6-well plate, seed $1.2x10^5$ Huh-7.5 cells per well in 4 ml media, and allow the cells to adhere and grow (**Note 6**).
2. Inoculate the samples with J6/JFH HCVcc at a low multiplicity of infection in 2 ml DMEM + 3% FCS; allow the infection to proceed for 72 h (**Note 7**).
3. To fix, remove the media from each well was and rinse with 1 ml cold PBS. Remove the PBS and add 4 ml of ice-cold methanol to each well and incubate at room temperature (RT) for 15 min (**Note 8**). Following

proper fixation virus particles will be inactivated and the samples can be handled on the bench.

3.4 Immunostaining

Immunostaining is the most commonly used labelling strategy, but labelling can also be achieved through genetically-encoded tags such as fluorescent proteins. In either case, an optimal label will be highly specific to the target and provide a strong fluorescent signal. Careful consideration should also be given to the possible pitfalls of any given labelling method. For example, some fluorescent proteins have a tendency to multimerize causing aberrant clustering of the target protein [12]; whereas use of primary and secondary antibodies, creates a physical separation between the target protein and the fluorescent signal. Discussions on the various properties of different labelling methods can be found here [13-16].

1. Rinse each well twice with 4 ml PBS to wash away methanol and rehydrate the samples (**Note 9, 10**).
2. Retrieve the coverslips in to a fresh 6 well plate, containing 2 ml PBS per well.
3. Block the samples with 2 ml PBS + 1% BSA for >90 min (**Note 11**).
4. Centrifuge primary antibody (anti-NS5A 9E10) at full speed in a microcentifuge for 5 min to pellet any large aggregates of antibody. Dilute the antibody to 100 ng/ml in PBS + 0.5% BSA. Add 1 ml of diluted antibody per well and incubate for 30 min at RT with gentle agitation.

5. Aspirate and discard the primary antibody and wash the sample in 2 ml PBS at RT for 5 min with gentle agitation.
6. Repeat step 5, twice.
7. Centrifuge secondary antibody (anti-mouse IgG Alexa Flour 647) (**Note 12**) as above. Dilute to 2 µg/ml in PBS + 0.5% BSA, pass the prepared solution through a 0.2 µm syringe filter prior to adding 1 ml to each well (**Note 13**). Incubate for 120 min at RT with gentle agitation.
8. Perform three PBS washes as described in step 5.
9. Incubate samples with 1 ml of 2 µg/ml DAPI diluted in PBS for 10 min to counterstain nuclear DNA.
10. Post-fix the samples in PBS + 1% formaldehyde for 10 min at RT with gentle agitation (**Note 14**).
11. Perform two PBS washes as described in step 5.
12. Store the samples in 2 ml PBS at 4°C until ready to use, they will be stable for 3-7 days.

3.5 Imaging

The imaging presented here was performed on a commercial SRM system, however the procedure and subsequent image reconstruction are capable of producing high-quality super-resolution images on less specialized microscopes. This method of SRM can be performed on any widefield microscope with the following specifications: i) a high magnification/high numerical aperture objective (e.g. 60-100 X oil objectives); ii) a sensitive, low noise camera (e.g. most EMCCD or sCMOS cameras); iii) a strong fluorescent light source, ideally a laser, although a powerful LED source is

sufficient for SRM, albeit with reduced resolution [8]. Systems matching these minimal requirements are common, making the approach outlined here accessible to most, if not all, researchers.

1. Transfer a coverslip with prepared sample in to an AttoFluor chamber. Ensure the coverslip is sitting face-up within the central lip and carefully tighten the chamber to seal. Add 1.5 ml of imaging buffer to the chamber (**Note 15**).
2. Place a fresh clean 25 mm coverslip on top of the buffer in the chamber, taking care to avoid the formation of air bubbles. Press down gently on the coverslip to create a loose seal and remove excess buffer with a pipette (**Note 16**).
3. Turn on the microscope 30-60 min before acquisition (**Note 17, 18**).
4. Place a small drop of oil on the 100X objective.
5. Place your sample securely on the microscope sample holder and carefully find focus, using the DAPI reference channel to observe the sample.
6. Select the 642 nm laser for Alexa Fluor 647 excitation and the appropriate emission filter.
7. Find an appropriate region of interest by searching the sample at low laser power.
8. Engage the microscope focus lock (**Note 19**) and leave the sample to rest for 5 min (**Note 20**).
9. Set the microscope to acquire widefield images using epifluorescence illumination.

10. Take diffraction-limited reference images of the field of view using the DAPI and Alexa Fluor 647 channels. This will provide a comparison for the super-resolution image.
11. Acquire 60,000 frames at 30 frames per second (33 ms exposure time) at 100% 642 nm laser power (approximately 2 kW/cm2). The combination of imaging buffer and high intensity illumination will induce the Alexa Fluor 647 dye molecules to photoswitch; the sample will appear to start ‘twinkling’ as the individual dyes blink on and off stochastically. Examples of typical fluorescent blinking can be seen in these video tutorials [17, 18]. It is this property that allows mathematical reconstruction of the super-resolution image (**Note 21, 22, 23, 24**).
12. Repeat steps 9-13 until an appropriate number of fields have been acquired.

3.6 Image reconstruction.

Localization SRM uses complex data analysis algorithms to extract super-resolved image reconstructions from conventional microscopy data. There is a bewildering variety of software packages for performing this task, each with their own relative flaws and strengths, as outlined in a recent comprehensive comparison [7]. Here, we outline the use of Super-Resolution Radial Fluctuations analysis (SRRF, pronounced ‘surf’), this is available as a plugin for the commonly used image analysis platform ImageJ/FIJI [10, 11]. SRRF is capable of improving resolution across a range of sample types, microscope

specifications and data qualities [8]. For these reasons, it is a good place to start, particularly for novice users (**Note 25**).

1. Install SRRF in ImageJ/Fiji (**Note 26**).
2. Ensure your dataset is in an appropriate format (i.e. .tif, .czi, .nd2, or .nji) and open the dataset in ImageJ/Fiji (**Note 27**).
3. Estimate sample drift in the data (in ImageJ/Fiji select Plugins -> NanoJ-SRRF -> Estimate drift), using the default values for Time averaging and Maximum expected drift. The drift estimate function will generate a drift table that must be saved, and then applied in the next step.
4. Perform image reconstruction analysis: In ImageJ/Fiji, select Plugins -> NanoJ-SRRF -> SRRF analysis, set each of the analysis parameters to the default values stated in the dialogue box, select “Do Drift-Correction”. To run the analysis, click “OK” and, when prompted, select the relevant drift correction table (generated in the previous step) (**Note 28**).
5. Once the analysis is completed, SRRF will provide a super-resolved reconstructed image as a 32bit tif file, which can be saved and processed as desired. The default reconstruction setting uses a magnification factor of 5; in the examples provided here, the reconstructed image has a pixel size of 20 nm (i.e. 5 times smaller than the 100 nm pixels in the raw data) (**Note 29**).

Example SRRF reconstructions of HCV infected cells stained with anti-NS5A are shown in **Figure 2** alongside conventional diffraction limited images as a

comparison. HCV induces extensive remodeling of the endoplasmic reticulum to produce a structure known as the membranous web, where genome replication and virus assembly are thought to occur [19, 20]. The ultrastructure of the membranous web has previously been studied using EM, as conventional microscopy does not provide a sufficiently high resolution. The SRRF reconstructions in **Figure 2** provide fine details of this convoluted membrane structure, which are not apparent in the conventional images. Vesicular structures ranging from 200-400 nm in diameter are clearly visible, these likely represent double membrane vesicles and/or lipid droplets [20-22].

The protocol presented here provides an example of how SRM can be used to visualize the basic biology of HCV infected cells. More advanced variations on this procedure may include multi-color SRM of viral and/or host components; whilst this presents technical challenges, particularly in properly aligning the two SRM channels, there are a variety of technical articles to provide guidance [23-25]. Another advantage to SRRF is its ability to extract super-resolution reconstructions from live-cell microscopy images. To attempt this, viral and/or host components will need to be tagged with photoswitching fluorescent proteins (such as mEOS) or dye accepting peptides [12, 26]. Users wishing to perform live-cell SRM should consult the original SRRF publication [8] and the associated online resources at https://bitbucket.org/rhenriqueslab/nanoj-srrf.

4. Notes.

1. At this stage, the coverslip is prone to floating on top of the water. If this occurs use tweezers to gently resubmerge.
2. More thorough cleaning methods can be employed if necessary. For alternative methods see these articles [27, 28].
3. Fine pointed tweezers and a 19G syringe, with the tip bent in to a slight hook, are ideal for manipulating the coverslips.
4. Other coverslip holders are also suitable, however, this particular brand easily fits within a 100 ml beaker. It is also sufficient to manually hold each coverslip with fine pointed tweezers during the coating procedure.
5. As an alternative, poly-L-lysine (PLL) coating also promotes cell adhesion.
6. A low/medium density of cells is ideal for high-resolution microscopy.
7. It is ideal to have a mixture of both infected and uninfected cells; this can assist the discrimination of signal over background.
8. The manner of fixation is an important parameter and may need to be optimised, taking consideration of the characteristics of each method. Methanol fixation dehydrates the sample leading to denaturing and precipitation of proteins and some lipid extraction, resulting in simultaneous fixation and permeabilization of the sample. However methanol fixation is not appropriate for imaging of fluorescent proteins. Aldehyde-based fixatives (e.g. 4% EM grade formaldehyde in PBS) covalently cross-link juxtaposed proteins resulting in a fixed meshwork. The addition of low percentage gluteraldehyde (<1%) may be necessary to immobilise membrane-associated proteins. Also

detergent treatment (e.g. 0.1% Triton X-100 in PBS) will be necessary to permeabilize aldehyde fixed cells prior to antibody staining of intracellular compartments. More detailed discussion of fixation methods can be found here [29, 30].

9. In all subsequent steps the samples should be treated with care, aspirate and apply buffer from the edge of the well avoiding contact with the coverslip.
10. During immunostaining buffers should be passed through a 0.2 or 0.45 µm filter prior to use. This is not to ensure sterility but to minimise the introduction of dust and precipitates.
11. The blocking step is critical to producing a high-quality sample. It serves to minimise the non-specific binding of antibodies and may need to be optimised. BSA blocking is normally sufficient and concentrations of up to 5% are commonly used. Blocking for extended periods or overnight may also help.
12. Alexa Fluor 647 is an excellent and commonly used dye for localization SRM. However, if performing dual labelling it should be noted that Alexa Fluor 647, and related far-red dyes such as Cy5, exhibit spurious fluorescence in the near-red (i.e. ~550-600 nm) and, therefore, should not be combined with red dyes [31]. In this case, a green dye such as Alexa Fluor 488 would be a more appropriate partner.
13. Syringe filtration is an additional precaution to eliminate secondary antibody aggregates, which can be particularly detrimental to sample quality.

14. This optional post-fix step can preserve the sample and minimise gradual dissociation of antibodies.
15. The buffer is an optimised photoswitching buffer for inducing blinking in fluorescent dyes [32]. It contains an oxygen scavenging system (Oxyrase) and a reducing agent (MEA) to promote most commonly used dyes to photoswitch, but may not be suitable for some fluorescent proteins.
16. Placing a coverslip on top of the buffer minimises exposure to fresh oxygen in air and ensures optimal imaging conditions for hours. However, avoid a perfect airtight seal on top of the chamber as this risks generating a slight vacuum, which can crack the sample coverslip.
17. High quality SRM reconstructions require tens of thousands of microscopy images to be acquired. One of the biggest challenges is the lateral drift (x-y plane) of the sample during acquisition. If possible, the microscope should be turned on 60 min before the start of the experiment and the sample should be left on the stage for 15 min before imaging to allow warm-up and equilibration. Some algorithms (including SRRF, used here) can calculate and correct lateral drift using the raw imaging data. However, this requires structures that remain visible throughout the entire image sequence, which is not always the case. As an additional precaution, a low density of fiducial markers can be added to the sample to assist accurate drift calculation and correction. Fiducials are bright fluorescent markers that remain

visible for the duration of the experiment, they are also necessary when aligning channels during multi-colour SRM [17, 32, 33].

18. Many commonly used microscopes have an additional magnification lens that can be introduced in to the light path to provide modest additional magnification; if available, this should be used. This example employs a 1.6 times magnifying lens, which, when combined with a 100 X objective this yields an image pixel size of 100 nm and results in superior data for image reconstruction.
19. Drifting focus, like lateral movement of the sample, is detrimental to data quality over long acquisitions. Many commercial systems are fitted with a focus-lock system that will maintain focus for many hours.
20. Resting the sample for a few minutes will allow the stage to settle and further minimise lateral drift during acquisition.
21. In epifluorescence illumination the excitation light passes through the entire depth of the sample, consequently the resulting image includes some out-of-focus emissions from above and below the focal plane; this will moderately decrease the resolution achieved (by corrupting the precision by which single-molecules can be localized). It is possible to decrease fluorophore excitation outside of the focal plane using alternative illumination modalities such as such as Highly Inclined Thin Illumination (HiLo) or Total Internal Reflection Fluorescence (TIRF). For instance, TIRF allows selective illumination the basal part of the cell (within 100-200 nm of the coverslip) [25, 34].
22. For true localization microscopy, the signals from individual dye molecules should be clearly discernable from adjacent signals.

However, for densely labelled structures it can be difficult to achieve sparsely distributed signals. Therefore, in this protocol we use a versatile reconstruction algorithm (SRRF) that can extract super-resolution images from sub-optimal data, albeit with reduced resolution.

23. The duration of the acquisition, imaging speed and laser intensity can be empirically tailored for the user's experimental and technical conditions [34, 35]. However, for a classical localization microscopy dataset we recommend a high number of frames (50,000-100,000), at maximum illumination power and maximum frame rate.
24. The rate of blinking by Alexa Fluor 647 may reduce during the acquisition, resulting in very sparse signals. If this occurs, low-level illumination with the 405 nm laser (for instance, 0.01-1%) can reactivate the dye molecules to increase the density of signals.
25. Alternatively, ThunderSTORM is an excellent reconstruction software that works particularly well on sparsely separated dye signals [36].
26. The original SRRF software package is provided in the supplementary materials of Gustafsson *et. al.* [8]. Up-to-date versions and user guides can be found here: https://bitbucket.org/rhenriqueslab/nanoj-srrf.
27. Given the high intensity illumination used to acquire the data, the first few thousand frames of the image series are often saturated by the high signal. This saturated data is not suitable for reconstruction; either remove these frames from the image stack or direct SRRF to crop them during analysis.
28. SRRF does not perform typical localization microscopy, but uses sophisticated image analysis to estimate the distribution of dye

molecules across a sample. It is a particularly versatile reconstruction method as it can extract super-resolved images from difficult datasets with high densities of fluorescent signals. SRRF exploits both the spatial and temporal characteristics of the emitted fluorescence across an image series to extract information about the underlying fluorophore distribution. An in-depth description of the SRRF method and parameters can be found here [8] and various online resources can be found here: https://bitbucket.org/rhenriqueslab/nanoj-srrf. If necessary the reconstruction process can be optimised to produce higher quality images. If unsure about which parameters to choose for a given dataset, crop a small representative part of the data (including different fluorophore densities and/or structures of interest) and run the Parameter Sweep macro, available for download with a user guide from https://bitbucket.org/rhenriqueslab/nanoj-srrf/downloads/.

29. The time taken to analyse the image will depend on the size of the data set and the specifications of the analysis computer. SRRF exploits the GPU processors found in computer graphics cards, as such SRRF will run more quickly on computers with powerful graphics engines. On a moderately powerful desktop computer, a large, 512 by 512 pixel, image series is likely to take tens of minutes to analyse.

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Figure Captions.

Figure 1. The principles of localization SRM.

A. Consider a nanoscale object decorated with fluorescent dye. **B.** When viewed by conventional microscopy the signals from closely apposed dye molecules merge and their nanoscale distribution is lost. **C.** In localization microscopy the dye molecules are induced to “blink” on and off stochastically, such that the signals from individual molecules can be viewed in isolation. Mathematical analysis of these signals allows the true location of the dye molecules to be estimated with high precision. Through prolonged imaging it is possible to locate and map sufficient numbers of molecules such that the ultrastructure of the nanoscale object can be reconstructed.

Figure 2. Super-resolution reconstructions of HCV infected Huh-7.5 cells. Samples and images were generated as described in the text. The “Red Hot” look up table has been applied to the images and brightness and contrast settings altered slightly for display purposes. **A.** Conventional diffraction-limited (left) and super-resolution (right) images of HCV infected cells stained with anti-NS5A. **B.** Enlarged views of the inset areas (i and ii) shown in A, conventional (left) and super-resolution (right) images are shown for each. The super-resolution images clearly show looping and vesicular

structures (white arrowheads) that likely represent the HCV-induced membranous web. These structures are barely discernable in the conventional microscopy images. **C.** Two additional representative super-resolution reconstructions of HCV infected cells. Scale bars 10 µm (**A** and **C**) and 2 µm (**B**). The images shown here achieved a resolution of 75-100 nm, conventional microscopy typically achieves 200-300 nm.