Imaging biological samples by integrated differential phase contrast (iDPC) STEM technique

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ABSTRACT

Scanning transmission electron microscopy (STEM) is a powerful imaging technique and has been widely used in current material science research. The attempts of applying STEM into biological research have been going on for decades while applications have still been limited because of the existing bottlenecks in dose efficiency and non-linearity in contrast. Recently, integrated differential phase contrast (iDPC) STEM technique emerged and achieved a linear phase contrast imaging condition, while resolving signals of light elements next to heavy ones even at low electron dose. This enables successful investigation of beam sensitive materials. Here, we investigate iDPC-STEM advantages in biology, in particular, chemically fixed and resin embedded biological tissues. By comparing results to the conventional TEM, we have found that iDPC-STEM not only shows better contrast but also resolves more structural details at molecular level, including conditions of extremely low dose and minimal heavy-atom staining. For thick sample sections, iDPC-STEM is particularly advantageous. Unlike TEM, it avoids contrast inversion canceling effects, and by adjusting the depth of focus, fully preserves the contrast of relevant features along with the sample. In addition, using depth-sectioning, iDPC-STEM enables resolving in-depth structural variation. Our work suggests that promising, wide and attractive applications of iDPC-STEM in biological research are opening.

KEY WORDS

Biological sample; Contrast; Integrated differential phase contrast; Scanning transmission electron microscopy; Thick section;

1 INTRODUCTION

2 The invention of electron microscope has been a great millstone for human to
3 observe the micro-world and investigate the nature at nano and atomic scale. Various
4 electron microscopy (EM) techniques, e.g., transmission electron microscopy (TEM),
5 scanning electron microscopy (SEM), scanning transmission electron microscopy
6 (STEM) and so on, have been developed in the past seventy years. Vast amount of
7 structural and dynamics information from various diverse materials have been obtained
8 by electron microscopy. However, the application of EM to study biological specimen
9 has been delayed for many years due to four major limitations. First, the vacuum of
10 specimen chamber of electron microscope is in conflict with the need of water for live
11 biological sample; second and more important, biological samples are highly sensitive
12 to electron radiation (Baker and Rubinstein, 2010; Glaeser, 1971); third, the thickness
13 of interest of biological samples is normally beyond the suitable penetration depth of
14 the electron; and fourth, biological samples are mainly consisting of light elements (C,
15 H, O, N, P and S), resulting a relative low contrast with normal EM techniques.

16 To overcome these challenges for biological TEM, large number of efforts have
17 been performed in the past. The first attempt was to develop chemical fixation and
18 dehydration protocols and embed biological sample into resin by trying to keep close
19 to native state. The ultrathin section of resin embedded biological sample can be imaged
20 directly by normal TEM. Heavy metal staining (osmium oxide, uranyl acetate or lead
21 citrate) was always used to enhance the TEM contrast of the resin section. With this
22 procedure, biologists obtained great achievements by discovering fruitful information
23 of cellular ultrastructures in the last century. The second attempt was to develop cryo-
24 fixation protocol and the technique of cryo-electron microscopy (cryo-EM) (Adrian et
25 al., 1984; Dubochet et al., 1988). The cryo-vitrified biological samples keep their most
26 natural state and can be imaged to atomic resolution by cryo-EM (Nakane et al., 2020;
27 Yip et al., 2020). Nowadays, cryo-EM has become one of the major biophysical
28 techniques to study 3D structures of bio-macromolecules, giving a great impact on the
29 life science research.

Introducing other EM techniques that are already available for materials science research into biological research has been always welcome with the aim to compensate for the current biological TEM techniques shortcomings and provide new useful information of biological samples. As a fully proved successful technique in characterizing materials at micro, nano, and atomic scales for a vast variety of different samples, STEM technique has been widely utilized and nowadays become one of the major high-resolution imaging tools in materials science. STEM imaging mode is obtained with an electron beam that is finely focused to a small probe at the plane of the specimen and then scanned over it. Depending on the convergence angle of the beam, the depth of focus of STEM can easily be altered to become longer than a given sample thickness. The probe scans the sample point by point, and transmitted electrons are collected and counted for each point by STEM detectors in the far field. The electrons scattered to different angles can produce different images. The brightfield (BF) detector collects electrons scattered within the convergent angle of the beam, whereas annular darkfield (ADF) detectors collect electrons scattered outside of the beam convergent angle regions, often to relatively high angles (HA) of ADF (see various literatures summarized in text books (Reimer and Kohl, 2008; Spence, 2013) and recent analytical in depth analysis (Bosch and Lazić, 2015; Lazić and Bosch, 2017)).

Applying STEM in biological research was originated only couple of decades ago (Colliex and Mory, 1994). Compared to TEM, STEM generate variety of signals, which can be used for biomacromolecule mass quantitative measurement, elemental mapping, and multi-signal imaging (Aronova and Leapman, 2012; Colliex and Mory, 1994; Sousa and Leapman, 2012). With the Z^2 - contrast feature of (HA)ADF-STEM favoring heavy elements (Bosch and Lazić, 2015, Lazić and Bosch, 2017), STEM has the unique advantage of visualizing nanogold particles (1.4 nm in diameter) embedded in the biological resin section (Sousa et al., 2007), which is very important for the immune-EM research. The beneficial aspect of STEM in electron tomography of resin embedded biological section has also been investigated (Aoyama et al., 2008; Hohmann-Marriott et al., 2009; Sousa and Leapman, 2012; Yakushevskaya et al., 2007). Compared to TEM

1 tomography, STEM tomography could generate a tomogram with better contrast and
 2 better signal-to-noise ratio (SNR) (Yakushevskaya et al., 2007). In addition, more
 3 importantly, some STEM techniques have a large depth of focus, no oscillation of
 4 contrast transfer function (CTF) (e.g. HAADF-STEM) and can achieve dynamic
 5 focusing, which endows STEM tomography a unique advantage of studying thick (1
 6 μm) biological sections (Aoyama et al., 2008). Considering the multi-scattering effect
 7 of electrons, the resolution and contrast of STEM tomography of thick section could be
 8 further improved by changing dark field to bright field (Hohmann-Marriott et al., 2009).
 9 The attempts of applying STEM tomography to frozen-hydrated biological sample were
 10 also performed in recently years (Rez et al., 2016; Trepout, 2020; Wolf and Elbaum,
 11 2019; Wolf et al., 2014), which turned to be useful to detect metabolic ions in
 12 biomacromolecules (Elad et al., 2017), study mitochondrial calcium stores physiology
 13 (Wolf et al., 2017) and dynamics of ferritin crystallization (Houben et al., 2020).
 14 Besides collecting tilt series like conventional electron tomography, STEM offers
 15 another possibility of tomography by depth section (Bosch and Lazić, 2019; Trepout et
 16 al., 2015).

17 Although many above efforts of trying STEM imaging in biological applications
 18 have been performed, this technique has not yet been widely used in the current
 19 biological research. Although (HA)ADF-STEM could yield a good, interpretable
 20 contrast, the number of electrons collected in the dark field is several orders of
 21 magnitude smaller than that in the bright field, effectively making (HA)ADF-STEM
 22 extremely dose inefficient with bad signal to noise ratio (SNR) and not suitable for dose
 23 sensitive biological samples. Furthermore, the contrast of (HA)ADF-STEM imaging is
 24 proportional to the square of elemental Z value (Lazić and Bosch, 2017), causing the
 25 biological light elements (C, H, O, N, etc.) to be less visible when imaged next to
 26 heavier ones (Si, P, S etc.) or invisible next to heavy ones (Ga, Au, Os, Ur, Pb, etc.).
 27 The image contrast of (annular) bright field ((A)BF) STEM is nonlinear, difficult to
 28 interpret, and the CTF of (A)BF-STEM also suffers from the shortcoming of
 29 oscillations (Lazić and Bosch, 2017) the same way TEM does, which is why it does not

1 show any superior advantage over the conventional TEM.

2 An appropriate STEM imaging technique for biological samples should generate
3 linear contrast with positive definite CTF, including all frequencies and using all
4 possible available electrons. Such a technique has been introduced, called Integrated
5 Differential Phase Contrast STEM (iDPC-STEM) (Lazić et al., 2016; Yucelen et al.,
6 2018). The iDPC-STEM is based on the detection of center of mass (COM) or center
7 of illumination of the beam in the detector plane, which can be effectively accomplished
8 already with 4-quadrant segmented detectors. The COM signal linearly maps the
9 electric *vector* field of the sample and an integration (inverse gradient) of this vector
10 map yields a *scalar* electrostatic potential field of the sample, resulting in a final image
11 with the linear phase contrast. Theoretical calculations proved the CTF of iDPC-STEM
12 is definite positive without any oscillations, and frequency transfer includes low
13 frequencies. Therefore, using iDPC-STEM technique, light and heavy elements can be
14 successfully imaged together (Gauquelin et al., 2017; Nahor et al., 2018; Song et al.,
15 2019; Yucelen et al., 2018), including hydrogen atoms (de Graaf et al., 2020). In
16 addition, the integration step of iDPC-STEM makes a natural suppression of the non-
17 conservative part of the noise, i.e. only conservative field (here electric field) can be
18 integrated. Due to this, a superior SNR is obtained (Lazić and Bosch, 2017; Lazić et al.,
19 2016) and a low dose imaging is enabled for the carbon-based and other beam sensitive
20 materials like zeolites (Shen et al., 2020b; Shen et al., 2021) and metal organic
21 frameworks (MOFs) (Shen et al., 2020a) with quality better than the one offered by
22 conventional TEM.

23 Although iDPC-STEM has been successfully and widely used in material science
24 as listed above, its advantages for biological samples have not been explored yet. In this
25 work, for the first time, we demonstrate ability of iDPC-STEM to image chemically
26 fixed and resin embedded biological tissue and cell samples. We found that, compared
27 to TEM images, iDPC-STEM images could not only show better contrast but also
28 resolve more cytosolic details in molecular resolution on the conditions of either low
29 dose or minimal heavy atom stains. Furthermore, for the thicker sections (400-600 nm

1 thickness), iDPC-STEM is superior and can resolve more structural details where the
2 conventional TEM suffers from contrast inversion canceling effects (the defocus
3 gradient induced signal fading due to thickness) and fails. For completeness, an initial
4 trial of depth-sectioning of a thick biological section has been performed showing
5 capability to resolve in-depth structural information.

1 MATERIALS AND METHODS

2 Theoretical consideration of the beam convergence semi-angle

3 A focused electron beam in STEM, called the probe, takes different shapes along
 4 the direction of propagation depending on electron energy, beam convergence semi-
 5 angle and aberrations introduced by forming condenser lenses. For most practical
 6 purposes, all aberrations except spherical aberration can be corrected to acceptable
 7 values by well-designed cylindrically symmetric column, proper alignment and
 8 stigmation correction. The regular STEM probe along the electron propagation shows
 9 a spindle-like shape (**Figure 1a**). Most intensity of the probe is concentrated within the
 10 region of the spindle, which interacts the strongest with the sample electric field and
 11 contributes dominantly to the signal at the detector and, therefore, the contrast of STEM
 12 images (Bosch and Lazić, 2019). With fixed spherical aberration, the size of STEM
 13 probe depends directly on the convergence semi-angle α that is determined by the
 14 condenser lens aperture. The width of the probe determines the x-y resolution of STEM
 15 imaging, and the length of the probe determines the depth of focus of STEM imaging,
 16 the z resolution in depth-sectioning mode. Within the critical condition (see description
 17 below) the larger the convergence semi-angle, the smaller the probe width and the probe
 18 length are, and vice versa. For a better resolution and reduced depth of focus, a larger
 19 convergence semi-angle is needed (**Table 1**).

20 There are two STEM imaging modes used in this work (**Figure 1b**). The first mode
 21 involves the depth of focus longer than the sample thickness. In this mode (**Figure 1b**,
 22 top) the sample can be considered thin with a high level of accuracy (Bosch and Lazić,
 23 2019), and the image formation can be fully described with the thin sample STEM
 24 theory (Bosch and Lazić, 2015). In this mode the in-focus image is obtained when the
 25 sample is located at the position of the beam central cross-over (the disc of least
 26 confusion), while a slightly defocused image is formed when the central position of the
 27 probe is apart above (over focus) or below (under focus) the sample. For iDPC-STEM
 28 and ADF-STEM the in-focus image will have the best contrast. Unlike that, for (A)BF-

1 STEM the most contrast will occur out of focus, for defocus value called Scherzer
2 defocus (Kirkland, 2010). The second imaging mode involves the depth of focus shorter
3 than the sample thickness (**Figure 1b**, bottom). In this mode the sample cannot be
4 considered thin, and the depth-sectioning of the sample takes place (Bosch and Lazić,
5 2019). By changing defocus, one could cover and image the full 3D volume of the
6 sample with the resolution in-depth determined by the length of the beam depth of focus
7 region (de Jonge et al., 2010).

8 When the spherical aberration (C_s) of the condenser system is not corrected the
9 smallest probe is defined with a critical convergence semi-angle, determined with
10 $\alpha_{max} = 1.34 \lambda / (C_s \lambda^3)^{1/4}$, where λ is relativistic wavelength of the electron, see
11 analysis by Kirkland (Kirkland, 2010) (Sec. 3.5.1). For the convergence semi-angles
12 smaller than this critical value the probe is practically the same in shape and size as for
13 corrected systems, while further increasing the convergence angle results in a
14 dramatically deteriorate shape of the probe in all three directions (**Figure S1**). In this
15 work, we used an aberration non-corrected microscope and carefully set up the
16 convergence semi-angle smaller than this critical value.

17 **Electron microscope set-up and imaging parameters**

18 In this work, all the electron microscopy images (both TEM and STEM) were
19 acquired using FEI Talos F200C (Thermo Fisher Scientific), an electron microscope
20 with a two-condenser lens, spherical aberration non-corrected ($C_s = 2.7$ mm) system,
21 operated at 200 kV. For the conventional TEM imaging, an equipped $4k \times 4k$ Ceta
22 CMOS camera and an objective aperture (100 μm) were used. For STEM imaging,
23 standard BF and HAADF detectors were used for usual (A)BF and ADF modes, as well
24 as standard segmented 4-quadrant detector for iDPC-STEM mode, and condenser
25 apertures (20 μm , 50 μm and 70 μm). All iDPC-STEM and TEM images were taken
26 and processed with the Velox software. For comparison of TEM and STEM images, the
27 pixel size for STEM has been selected as close as possible to the one applied in TEM.

28 The critical value of the convergence semi-angle of our electron microscope system

1 before probe starts deteriorating, based on formula given above and $C_S = 2.7 \text{ mm}$, is
 2 $\alpha_{max} = 7.4 \text{ mrad}$. Therefore, the dependency of the STEM resolution and depth of
 3 focus with respect to convergence semi-angles smaller than this critical value have been
 4 computed and shown in **Table 1**. With available apertures and condenser lens modes
 5 the two convergence semi-angles of 2.58 mrad (the corresponding depth of focus is 751
 6 nm) and 6.43 mrad (the corresponding depth of focus is 121 nm) have been selected for
 7 STEM imaging in this work.

8 **Electron dose calculation**

9 In TEM imaging, the total dose in $[e/\text{\AA}^2]$ units is calculated as total number of
 10 electrons (in $[e]$ units) passed per square area (in $[\text{\AA}^2]$ units) during exposure time,

$$11 \quad \text{total dose TEM } [e/\text{\AA}^2] = \text{dose rate } [e/\text{\AA}^2/\text{s}] \cdot t_{exp}[\text{s}] , \quad (1)$$

12 where the dose rate is beam current per unit area (beam current density). The beam
 13 current, usually calibrated and read in $[pA] = [pQ/s]$ is converted into $[e/s]$ units,
 14 using $1.00 [Q] = 1.00/(1.60 \cdot 10^{-19})[e] = 6.24 \cdot 10^{18}[e]$, before dose rate is
 15 computed, yielding $1.00 [pA] = 6.24 \cdot 10^6 [e/s]$. The TEM exposure time t_{exp} is
 16 measured in seconds.

17 In STEM, the total dose in $[e/\text{\AA}^2]$ units is calculated as total number of electrons
 18 passed the total field of view (FOV) area (full image area) after the full scanning frame
 19 is finished. This is the same for all type of STEM including iDPC-STEM, yielding

$$20 \quad \text{total dose STEM } [e/\text{\AA}^2] = \frac{\text{beam current } [e/s] \cdot t_{frame} [s]}{FOV^2 [\text{\AA}^2]} , \quad (2)$$

21 where the beam current, usually calibrated and read in $[pA] = [pQ/s]$ is converted
 22 into $[e/s]$ units, as above. The total scanning frame time t_{frame} is measured in seconds,
 23 while the total image field of view is given in $[\text{\AA}]$. The full frame time t_{frame} is the
 24 time spent per pixel, called the dwell time t_{dwell} , multiplied by the number of pixels
 25 visited by the beam. Assuming that $N \times N$ image is acquired, $t_{frame} = N^2 \cdot t_{dwell}$ and

1 $FOV = N \cdot pixel\ size$, which further yields more practical formula

$$2 \quad total\ dose\ STEM\ [e/\text{\AA}^2] = \frac{beam\ current\ [e/s] \cdot t_{dwell}\ [s]}{pixel\ size^2\ [\text{\AA}^2]}, \quad (3)$$

3 Where number of pixels simply cancels out.

4 The *dwell times* are normally many orders of magnitude smaller than the *frame times*
5 (e. g. for $1k \times 1k$ image that is one million times smaller) and, therefore, naturally
6 measured in $[\mu s]$. Beam current is, for practical cases in biology, measured in $[pA]$.

7 Taking this into account the above formula, Eq. (3), further becomes

$$8 \quad total\ dose\ STEM\ [e/\text{\AA}^2] = \frac{6.24 \cdot beam\ current\ [pA] \cdot t_{dwell}\ [\mu s]}{pixel\ size^2\ [\text{\AA}^2]}. \quad (4)$$

9 With a quick computation check we see that $1\ pA$ beam current, $1\ \mu s$ dwell time with a
10 scanning pixel size of $2.5\ \text{\AA}$, produces the total dose of $1.0\ e/\text{\AA}^2$.

11 **Preparation of resin embedded tissue sample**

12 Two samples, one for the human hepatocellular carcinoma cell (HepG2) and
13 another for the mouse testis tissue, were selected in this work to prepare resin embedded
14 sections. Two sample preparation protocols, one with regular heavy atom staining
15 (Method 1) and second with minimal staining (Method 2) were performed, respectively
16 **(Figure S2)**.

17 For the protocol of regular heavy atom staining (Method 1), after euthanasia of a
18 mouse at $4^\circ C$, tissues were quickly dissected and fixed in the fixative solution
19 containing 2.5% glutaraldehyde for 48 hr. For HepG2 cells, the cells were collected and
20 washed 3 times with PBS and fixed with 2.5% glutaraldehyde at $4^\circ C$. All samples were
21 washed with PBS and further fixed with the buffer containing 1% OsO₄ for 2 hr. The
22 chemically fixed sample were dehydrated using graded ethanol solutions from 20% to
23 100% and embedded in Epon 812 resin (SPI Supplies of Structure Probe, Inc.).
24 Ultrathin sections of resin embedded tissue sample were obtained using ultramicrotome
25 UC7 (Leica Microsystems) and further stained using 2% uranyl acetate for 30 minutes
26 and then lead citrate for 10 minutes.

1 For the protocol of minimal heavy atom staining (Method 2), the procedures from
2 tissue dissection/cells collection, fixation and dehydration to resin embedding and
3 ultrathin sectioning were exactly same as Method 1. The only difference is that there
4 was no further post staining on the ultrathin sections.

5 **Experimental set-up to investigate iDPC-STEM technique**

6 In this work we set up four kinds of experiments to investigate iDPC-STEM
7 imaging technique and make comparisons with the conventional TEM. First, we
8 investigate image contrast of the regularly stained tissue sections by changing electron
9 dose. We determine the required doses for iDPC-STEM and TEM, respectively, for
10 which the optimal contrast was obtained. Then, while keeping the electron dose at
11 which the conventional TEM imaging shows a good contrast (for the regularly stained
12 sample), we switch to the minimally stained samples and compare the contrast between
13 TEM and iDPC-STEM again. Next, we increase the thickness of the section from
14 commonly used 70 nm to 200 nm, 400 nm, even up to 600nm and compare the contrast
15 and image quality between TEM and iDPC-STEM again. Finally, in-depth structural
16 information acquisition of the thicker section with iDPC-STEM depth-sectioning is
17 demonstrated.

18 **Quantitative measurement of contrast**

19 To qualitatively compare the contrast in the images obtained using different
20 techniques and conditions, a method of line profile over the area of interest is used
21 (**Figure S3**). First, the images are normalized according to the pixel variance (step 1).
22 Then the line profile is generated at the region of interest using Gatan Digital
23 Microscopy software (step 2). Finally, the contrast is computed as difference between
24 maximum and minimum intensity along the profile, normalized to the maximum (step
25 3).

26

1 RESULTS

2 iDPC-STEM contrast vs. TEM contrast with respect to electron dose

3 We make the first comparison between iDPC-STEM and TEM using the regularly
4 prepared thin (70 nm) section (Method 1) of resin embedded HepG2 cell. We set the
5 convergence semi-angle of iDPC-STEM to a higher value, 6.43 mrad in this experiment.
6 The corresponding depth of focus is 121 nm (**Table 1**), which is larger than the thickness
7 of the section, while resolution down to 3 Å is, in principle, possible. Thus, we followed
8 the first imaging mode of STEM (**Figure 1b**) and optimized the z-position of the section
9 to get the sharpest iDPC-STEM images with the best contrast. The original pixel size
10 of iDPC-STEM images was set to 1.05 nm and then resized to match the pixel size of
11 TEM images by FFT cropping algorithm. For TEM imaging, we set up an appropriate
12 magnification to image the same region of the section with a similar field of view (FOW)
13 and the final pixel size on the camera was 1.50 nm. We used a big defocus value of ~7
14 μm in TEM imaging to compensate the attenuation of CTF at the low frequency and
15 achieve a good contrast.

16 We kept the electron dose for each compared pair of iDPC-STEM and TEM images
17 exactly the same and took a series of micrographs with different electron doses starting
18 from 1 e/Å², 3 e/Å², 6 e/Å², 10 e/Å², 20 e/Å² up to 40 e/Å² (**Figure 2a**). At the relatively
19 low dose conditions (1 e/Å², 3 e/Å², 6 e/Å² and 10 e/Å²), by qualitative examination
20 iDPC-STEM imaging produces better micrographs with better contrast and better SNR.
21 The structural details of organelle membranes and, especially, the fine structures within
22 the mitochondrial inter-membrane space, are clearly visualized and resolved within
23 iDPC-STEM micrographs, while not in the corresponding TEM images. We stress here
24 the extreme case, using electron dose of only 1 e/Å², where the conventional TEM does
25 not generate enough contrast to resolve the ultrastructure of mitochondria, while iDPC-
26 STEM resolves the mitochondrial double membrane and crista.

27 For quantitative comparison, a line profile across the mitochondrial membrane has
28 been produced and the contrast calculated within each micrograph as explained in

1 Methods and **Figure S3**. The results are summarized in **Figure 2b**. With increase of the
2 electron dose the micrograph contrast of both TEM and iDPC-STEM imaging increases.
3 However, at corresponding doses, the iDPC-STEM contrast stays constantly above
4 (around twice) compared to the conventional TEM contrast, significant even at the
5 extreme low dose value of $1 \text{ e}/\text{\AA}^2$.

6 Beside the enhanced contrast, iDPC-STEM resolves more structural information in
7 comparison with TEM. We further investigated a pair of micrographs in **Figure 2a**,
8 taken at the relative high exposure dose of $40 \text{ e}/\text{\AA}^2$, where TEM should behave optimally.
9 The region of one mitochondrion (purple rectangle) and the region of one
10 mitochondrion-contacted endoplasmic reticulum (ER)-like organelle (green rectangle)
11 in each micrograph were enlarged in **Figure 2c**. We found that a pair of ATP synthase
12 are resolved on the cristae membrane in the iDPC-STEM micrograph while we could
13 not identify them in the TEM micrograph. In addition, we could observe two membrane
14 protein complexes located at the luminal side of the membrane (red arrow) of the ER-
15 like organelle, while they could not be resolved in the corresponding TEM micrograph.

16 To prove consistency and reproducibility, we repeated the electron dose experiment
17 for another HepG2 cell specimen. This reconfirmed the results (**Figure S4**).

18 **iDPC-STEM contrast vs. TEM contrast with respect to staining**

19 Biological resin sections contain mainly light elements, providing a limited
20 contrast in normal TEM. Therefore, heavy metal (Os, U and Pb) staining has always
21 been used to enhance the contrast. Nevertheless, this staining procedure is complicated,
22 involves human safety concerns, and also, with heavy staining, introduces presumable
23 structural artifacts. Therefore, the future imaging technique is demanded to operate with
24 less staining, while preserving and better resolving the ultrastructure. In our second
25 experiment, we investigate iDPC-STEM in this respect, if it could generate images with
26 good enough contrast for the sections less stained.

27 We tested two kinds of sections of HepG2 cell, one regularly prepared, using
28 Method 1, and another with the minimal heavy atom staining, using Method 2. The

1 thickness of the sections was again 70 nm. The total electron dose for both TEM and
2 iDPC-STEM was set to 3 e/Å². This time, we selected two different convergence semi-
3 angles 6.43 mrad and 2.58 mrad for iDPC-STEM imaging. The corresponding depths
4 of focus are 121 nm and 751 nm, respectively (**Table 1**). These are both larger than the
5 thickness of the section and, therefore, the first imaging mode of STEM, shown in
6 **Figure 1b**, is taking place. The original pixel size of iDPC-STEM images was set to
7 1.43 nm and then resized to match the pixel size of TEM images by FFT cropping
8 algorithm. For TEM imaging, we set up an appropriate magnification and the defocus
9 value of ~ 7 μm to image the same region of the section with a similar field of view and
10 the final pixel size on the camera was 1.50 nm.

11 Comparing the sections prepared with Method 1 (high staining) and Method 2
12 (minimal staining), we found that the contrast of TEM micrograph significantly
13 decreased with the reduction of heavy atom staining. This is shown qualitatively in
14 **Figures 3a, 3b and 3c** and quantitatively, by line profile measurement in **Figure 3d**.
15 We found at the minimal staining condition (Method 2) the SNR of TEM micrograph
16 is quite low. However, at the same time, the reduction of contrast in iDPC-STEM
17 micrograph is much less, both observed qualitatively (**Figures 3a, 3b, and 3c**) and
18 quantitatively (**Figure 3d**).

19 For the section prepared with Method 1 (heavy staining), in consistency with the
20 previous experiment, the contrast of iDPC-STEM at 6.43 mrad is better than in TEM
21 (**Figure 3d**) and the ribosomes on the surface of rough ER are better resolved in
22 comparison with the corresponding TEM micrograph (indicated with the arrow in
23 **Figure 3b**). Furthermore, for the section prepared with Method 2 (minimal staining),
24 while TEM contrast deteriorated significantly (**Figure 3d**), the contrast of iDPC-STEM
25 at 6.43 mrad micrograph was still strong enough to resolve small vesicles (or protein
26 complex compartments) at the cytosolic space between the mitochondria and the
27 contact ER (indicated with the arrow in **Figure 3c**). This could by no means be observed
28 in the corresponding low contrast (low SNR) TEM micrograph (**Figure 3c**).

1 As mentioned, the two different convergence semi-angles (**Figure 3a**) have been
 2 applied for iDPC-STEM in this experiment. As expected from theoretical consideration
 3 (**Table 1**), because sample was thinner than the depth of focus in both cases, the larger
 4 convergence semi-angle produced sharper image. The smaller the convergence semi-
 5 angle, the larger the STEM probe becomes, resulting in reduced resolution and therefore
 6 sharpness (**Figure 3d**). At reduced resolution, resolving the double membrane feature
 7 of mitochondria (**Figure 3a**) naturally becomes harder, in some cases, worse than TEM.
 8 Therefore, selection of a proper convergence semi-angle is important for iDPC-STEM
 9 technique. Whenever sample allows (e.g. thinner) larger convergence angle should be
 10 used to enhance resolution and sharpness.

11 To prove consistency and reproducibility, we repeated the staining experiment for
 12 another HepG2 cell specimen. This reconfirmed the results (**Figure S5**).

13 **iDPC-STEM vs. TEM contrast and structural details for thick sections**

14 One of the important advantages of iDPC-STEM imaging is the ability to image
 15 thicker samples by applying smaller convergence angle of the beam (Bosch and Lazić,
 16 2019). The smaller convergence angle of the beam provides a larger depth of focus, that
 17 can easily become larger than the sample thickness. This enables the full projection of
 18 the sample electrostatic potential and enhancing the contrast for all the features
 19 extending along the sample in-depth direction.

20 In the next experiment, the section samples of mice testis tissues of different
 21 thicknesses, i.e., 200 nm, 400 nm and 600 nm, prepared using Method 1, have been
 22 investigated. The total exposure dose for both TEM and iDPC-STEM was set to $10 \text{ e}/\text{\AA}^2$.
 23 Two different convergence semi-angles 6.43 mrad and 2.58 mrad for iDPC-STEM
 24 imaging have been used, corresponding depths of focus as 121 nm and 751 nm,
 25 respectively (**Table 1**). The former is shorter, while the latter is longer than the thickness
 26 of the sections used in the experiment. The pixel size of iDPC-STEM images was set
 27 to 1.1 nm. For TEM imaging, we set up an appropriate magnification and the defocus
 28 value of $\sim 7 \text{ }\mu\text{m}$ to image the same region of the section with a similar field of view and

1 the final pixel size on the camera was 1.04 nm.

2 For the 200 nm thick section, conventional TEM still produces sharp image with a
3 reasonable contrast (**Figure 4a**, left). The current thickness is still within the mean free
4 path of biological section for 200 keV electron, which was measured to be 300 nm (Yan
5 et al., 2015). However, the effect of the focus gradient at the current thickness cannot
6 be avoided (Sun, 2018), affecting the resolution and contrast in TEM. For iDPC-STEM
7 both at 6.43 mrad and 2.58 mrad (**Figure 4a**, middle and right, respectively), good
8 quality micrographs have been produced, as good as the ones for 70 nm sections
9 (**Figure 2a**). A better contrast in comparison with the TEM image has been obtained,
10 qualitatively as well as quantitatively (see **Figure 4d**). Due to comparable depth of focus
11 at 6.43 mrad (121 nm) with the sample thickness (200 nm), the image contrast and
12 resolution of iDPC-STEM at 6.43 mrad is better than that of iDPC-STEM at 2.58 mrad.

13 For the section of 400 nm thickness, the quality of TEM micrographs deteriorate,
14 both due to inelastic scattering of electrons and defocus CTF canceling with most of
15 high frequency contrast lost. As the result, many fine membrane structures can no
16 longer be resolved (**Figure 4b**, left). However, for iDPC-STEM both at 6.43 mrad and
17 2.58 mrad good contrast micrographs are obtained, resolving many fine structural
18 details of the membrane (**Figure 4b**, middle and right). Quantitatively this is also
19 confirmed (**Figure 4d**). As expected, the image contrast of iDPC-STEM at 6.43 mrad
20 became worse than what was obtained for the 200nm thickness. This is because the
21 thickness of 400 nm is now noticeably larger than the depth of focus of 121 nm and the
22 image contrast is influenced by contributions from the regions below and above the
23 central part of the probe (**Figure 1b**).

24 For even thicker section, of 600 nm thickness, the quality of TEM micrograph
25 deteriorates further, for the same reasons as explained above, becoming practically
26 useless (**Figure 4c**, left). Only the low frequency contrast features, like membrane
27 shapes, are resolved. iDPC-STEM at the other hand, is barely affected and deteriorated
28 only minorly. Besides the shape, detailed membrane ultrastructure is resolved from the

1 micrographs, both at 6.43 mrad and 2.58 mrad. The image contrast obtained at 2.58
2 mrad by iDPC-STEM is now stronger than the one obtained by iDPC-STEM at 6.43
3 mrad (see quantitative comparison in **Figure 4d**). This is because the thickness of the
4 section (600 nm) is now significantly larger than the depth of focus of iDPC-STEM at
5 6.43 mrad (121 nm), while still smaller than the depth of focus at 2.58 mrad (751 nm).

6 To prove consistency and reproducibility, we repeated the thickness dependence
7 experiment for another mouse testis tissue specimen. This reconfirmed the obtained
8 results (**Figure S6**).

9 **Depth-sectioning of iDPC-STEM imaging for thick samples**

10 When the depth of focus of the electron beam is smaller than the sample thickness
11 (**Figure 1b**, bottom), besides electron tomography, to resolve the in-depth structural
12 information of a thick sample, a depth-sectioning method can be applied (Bosch and
13 Lazić, 2019; de Jonge et al., 2010). Here, we demonstrate this capability using iDPC-
14 STEM for biological sample. Although not optimal (ideally should be large > 30 mrad),
15 a convergence semi-angle of 6.43 mrad used here has been good enough to show some
16 structural variation at different depths.

17 We used Method 1 to prepare a resin embedded section of another mouse testis
18 tissue with the thickness of 600 nm. The total exposure dose for iDPC-STEM was set
19 to $3 \text{ e}/\text{\AA}^2$. The pixel size of iDPC-STEM images was set to 5 nm. We acquired a series
20 of iDPC-STEM images using different probe focal positions from over-focus (above
21 the sample) to under-focus (below the sample). The defocus step was chosen as 10 nm.
22 From this stack of the images, slices separated by 50 nm, representing the middle part
23 of the sample have been selected and shown in **Figure 5**. Zero defocus is set to be in
24 the middle of the sample. By investigating the slices, we found that the membrane
25 structure regions change at different depths. We indicated these regions in **Figure 5**
26 showing that the depth-sectioning did have an effect.

27 Note that the depth of focus with this convergence semi-angle is still quite large
28 (121 nm), meaning the in-depth resolution is very low. Therefore, revealing the full 3D

1 structural information is certainly not to be expected. Appropriate depth-sectioning can
 2 be provided with an aberration corrected probe, formed with the probe aberration
 3 corrected system (Borisevich et al., 2006). This allows larger convergent semi-angles,
 4 producing the probes smaller in all 3 directions (**Figure S1**, top row).

5

1 DISCUSSIONS

2 Attempt to apply STEM technology into biological research has been going on for
3 almost half a century, from molecular mass measurement (Colliex and Mory, 1994) to
4 tomography of biological sections (Aoyama et al., 2008; Yakushevskaya et al., 2007).
5 However, compared to TEM, the previously available STEM techniques had not shown
6 a significant advantage. In the present work, we investigated, recently emerged STEM
7 technique, iDPC-STEM (Lazić et al., 2016; Yucelen et al., 2018), which could solve the
8 low dose-efficiency issue of (HA)ADF-STEM and the non-linear contrast problem of
9 (A)BF-STEM.

10 Considering the significant radiation damage effect of biological specimen, we first
11 studied whether iDPC-STEM could yield good contrast of biological sections with
12 limited dose. We found that, from normal low dose condition of $40 \text{ e}/\text{\AA}^2$ to extreme low
13 dose condition of $1 \text{ e}/\text{\AA}^2$, iDPC-STEM could always generate reasonable contrast better
14 than conventional TEM. When the dose is below $3 \text{ e}/\text{\AA}^2$, discerning membrane
15 ultrastructure becomes difficult in TEM, while it is still clear in iDPC-STEM. We
16 consider two main reasons to explain this observation.

17 First, the vector data integration step in iDPC-STEM naturally reduces the noise,
18 as noise is a non-conservative vector field, while fully preserving the conservative
19 electric field vector which is our primary signal (Lazić et al., 2016). This increases
20 signal-to-noise ratio (SNR) of the final micrograph. The effect of adding the noise to
21 corresponding DPC-STEM components and the noise transfer to the final iDPC-STEM
22 image is demonstrated in **Figure S7**. Even when most of the signal spectrum is drown
23 into noise in the component images ($\text{SNR} \leq 1$), most of the spectrum stays above noise
24 ($\text{SNR} \geq 1$) in the final image. Such enhancement could not appear within TEM nor any
25 other conventional STEM technique.

26 Second, the contrast transfer function oscillations and low frequency transfer. For
27 heavy metal-stained resin-embedded section, as reported before, the contrast in TEM
28 micrograph is majorly contributed from the electron exit wave amplitude (Reimer and

1 Kohl, 2008). The electron wave phase information of resin-embedded section
 2 contributing from the cross-section difference between biological material and resin is
 3 small (Carlemalm and Kellenberger, 1982) and significantly faded by CTF oscillation
 4 effect in TEM. However, for iDPC-STEM, with positive definite CTF and full
 5 frequency transfer including low frequencies (Lazić et al., 2016) the result is similar to
 6 the effect of using phase plate in TEM (Danev and Nagayama, 2001; Danev et al., 2009;
 7 Edgcombe, 2016). This has been proven earlier (Lazić and Bosch, 2017) and in our
 8 experimental result shown in **Figure 2**. For the imaging result at $40 \text{ e}/\text{\AA}^2$, good contrast
 9 is observed for stained membrane structure in both TEM and iDPC-STEM, while the
 10 less stained membrane protein complexes could be only visualized using iDPC-STEM.

11 Besides dose efficiency, we investigated iDPC-STEM imaging of biological
 12 sections with the minimal staining. Since phase information is fully transferred in
 13 iDPC-STEM, a good contrast for less stained biological sections is expected. Our
 14 experimental data showed that while at the minimal stain condition the contrast of TEM
 15 micrograph significantly deteriorates iDPC-STEM micrographs do show good contrast
 16 and ultrastructural details at molecular level (**Figure 3**). This observation encouraged
 17 us to try iDPC-STEM technique on the unstained resin-embedded biological section,
 18 which was previously studied by HAADF-STEM (Carlemalm and Kellenberger, 1982).
 19 More interestingly, applying iDPC-STEM technique on the cryo-vitrified lamella of
 20 biological specimen would be a promising direction in the future.

21 Finally, we studied the influence of sample thickness on iDPC-STEM and TEM.
 22 The conventional TEM quality deteriorates rapidly with poor contrast with increase of
 23 sample thickness. There are two reasons for this phenomenon. The focus gradient
 24 resulting cancellation of CTF as the major one (Sun, 2018) and the significantly
 25 increased portion of inelastically scattered and multiple scattered electrons that
 26 contribute to the image background. The second becomes significant when the sample
 27 thickness is beyond the mean free path of accelerated electron (Egerton, 2011). Our
 28 experimental results confirm these effects. For the sample thickness of 200 nm, within
 29 the mean free path of 200 keV electron (Yan et al., 2015), the TEM micrograph still

1 yields good contrast with a bit reduced structural details, due to focus gradient. When
2 the sample thickness increases to 400 nm and 600 nm, this reduction becomes
3 significant, for most of the features, terminal.

4 For iDPC-STEM, when a proper semi-convergence angle is selected, the depth of
5 focus can be large enough to cover the whole range of the sample thickness, thus
6 avoiding the focus gradient problem. This is due to amorphous like nature of biological
7 samples that do not significantly alter and disturb the probe shape (Bosch and Lazić,
8 2019). As a result, iDPC-STEM is suitable to image thick samples, which we confirm
9 in our experimental investigation. With the convergence semi-angle of 2.5 mrad that
10 yields a depth of focus of 751 nm, the iDPC-STEM image contrast is constantly high
11 for all sample thickness cases (200nm, 400nm and 600nm), preserving detailed
12 membrane ultrastructure in the micrographs. This is because the 751 nm depth of focus
13 is comfortably larger than all these the sample thicknesses (**Figure 1b**, top row). With
14 the convergence semi-angle of 6.43 mrad that yields a depth of focus of 121 nm, the
15 contrast of iDPC-STEM is well preserved for the sample thickness of 200 nm
16 (comparable to 121 nm) while becoming slightly worse for the thicknesses of 400 nm
17 and 600 nm (**Figure 4d**). This is due to depth of focus becoming smaller and smaller
18 with respect to sample thickness (**Figure 1b**, bottom row). In this case, only part of the
19 central probe waist-length provides sharp signal of interest, while parts of the sample
20 above and below the beam waist interact with defocused parts of the probe and
21 contribute to the random-like background signal. This is what causes a drop in contrast,
22 although offers an opportunity of depth-sectioning. Note that, even though at certain
23 point high convergence semi-angle iDPC-STEM contrast becomes smaller than the
24 contrast obtained with small convergence semi-angle, it is still significantly better than
25 the contrast obtained with TEM.

26 In iDPC-STEM, a smaller convergence semi-angle needed to yield large enough
27 depth of focus, decreases the achievable in plane resolution (**Table 1**). Therefore, to
28 preserve high resolution for thick sample, a technique of depth-sectioning would have
29 to be performed (Bosch and Lazić, 2019). In the present work, we performed only a

1 proof-of-concept experiment using the semi-convergence angle of 6.43 mrad and depth
2 of focus of 121 nm (**Figure 5**). This depth of focus is still large and cannot yield a good
3 resolution in the vertical direction. To achieve better resolution in vertical direction,
4 there are two possible ways. One is to further increase the semi-convergence angle and
5 reduce the depth of focus, which can be only performed in a Cs-corrected microscope.
6 Another way is to combine depth sectioning and tomography to improve the resolution
7 in both lateral and vertical directions, which has been already reported for BF-STEM
8 (Trepout et al., 2015). This is out of the scope of this work.

9 Overall, we would like to conclude that iDPC-STEM technique shows a high
10 performance for studying resin-embedded biological section by providing fruitful
11 contrast in low dose imaging condition, suitable for minimal stains and owning unique
12 advantages for hick sample (**Table 2**), implying a promising future for its wide
13 application in the biological research.

14
15

AUTHOR CONTRIBUTIONS

F. S. and I. L. initiated and supervised the project. X. L. and X. H. performed all microscopy experiments. L. W. prepared resin embedded sections. X. L., X. H., I. L. and Y. D. analyzed experimental data. D. W, M. W. and L. Y. contributed to discussion of experimental design and data analysis. X. L., I. L. and F.S. wrote the paper.

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COMPLIANCE WITH ETHICAL STANDARDS

All authors declare that they have no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

COMPETING INTERESTS

Authors declare no Competing Financial or Non-Financial Interests.

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15

FIGURE LEGENDS

Figure 1. Theoretical diagram of STEM imaging. (a) Cross-section of STEM imaging system and the STEM probe. Enlarged vertical cross-section of the probe (middle) and illustrative probe model (right) are shown with the key parameters (convergence semi-angle α , resolution and depth) of the probe indicated. (b) Two main situations of STEM imaging in relation to the depth of focus vs. the sample thickness. Top: the depth of focus is larger than the sample thickness, the best in-focus image is obtained by placing the position of the sample just at the middle of the spindle-shaped probe (disc of least confusion). Bottom: the depth of focus is shorter than the sample thickness, different parts of sample are imaged with different focal position of the probe and the depth-sectioning can be performed.

Figure 2. Comparison between TEM and iDPC-STEM imaging at different electron dose. (a) TEM and iDPC-STEM micrographs acquired on 70 nm section of HepG2 cell with the total electron doses of 1 e/Å², 3 e/Å², 6 e/Å², 10 e/Å², 20 e/Å² and 40 e/Å², respectively. The pixel size for TEM is 1.50 nm. The original pixel size of iDPC-STEM was 1.05 nm, resized to 1.50 nm by FFT cropping algorithm. The convergence semi-angle of iDPC-STEM is 6.43 mrad. (b) Quantitative comparison of contrast between TEM and iDPC-STEM at different exposure doses. The contrast of each micrograph was measured using the line profile method (see **Figure S3**). For each data point, the measurement was repeated three times using different profile lines at different regions. (c) Zoom-in comparison between iDPC-STEM and TEM images at the dose of 40 e/Å². Regions of both iDPC-STEM and TEM images marked by the purple/green rectangles in (a), representing mitochondria (purple) and ER-like organelles (green), respectively, are zoomed in and arranged from the left (TEM) to the middle (iDPC-STEM) and from top (mitochondria) to bottom (ER-like organelles). The regions marked by red rectangles in iDPC-STEM images are further enlarged and shown at the right accordingly. In these enlarged images, the structure models of ATP synthase (EMDB entry EMD-11368) can be comfortably fit into the protein density on the mitochondrial cristae membrane (top right) and the membrane protein complexes

(marked by the red arrow, bottom right) can be visualized in the luminal space of the ER-like organelle.

Figure 3. Comparison between TEM and iDPC-STEM imaging for different amount of staining. (a) The sections of HepG2 cell prepared using Method 1 and Method 2, respectively. The thickness of the section is 70 nm. TEM images acquired with the defocus of $\sim 7 \mu\text{m}$ and the total exposure dose of $10 \text{ e}/\text{\AA}^2$. The iDPC-STEM images acquired with the total exposure dose of $10 \text{ e}/\text{\AA}^2$ and two convergence semi-angles 6.43 mrad and 2.58 mrad, respectively. The pixel size of TEM is 1.50 nm. The original pixel size of iDPC-STEM was 1.43 nm, resized to 1.50 nm by FFT cropping algorithm. (b) Regions marked by the green rectangles are zoomed in and compared. Left, TEM. Right, iDPC-STEM. The ribosome particles on the ER membrane are indicated by the red arrow in the iDPC-STEM image. (c) Regions marked by the purple rectangles are zoomed in and compared. Left, TEM. Right, iDPC-STEM. The interesting ultrastructures (vesicle or protein complex compartment) between mitochondrion and ER are indicated by the red arrow in the iDPC-STEM image. (d) Quantitative comparison of contrast between TEM and iDPC-STEM images for different stained sections. The contrast of each micrograph was measured using the line profile method (see **Figure S3**). For each data point, the measurement was repeated three times using different profile lines at different regions.

Figure 4. Comparison between TEM and iDPC-STEM imaging for different section thicknesses. The sections of mice testis tissues were prepared using Method 1 with different thickness of 200 nm (a), 400 nm (b) and 600 nm (c), respectively. TEM images (left) were acquired with the defocus of $\sim 7 \mu\text{m}$ and the total exposure dose of $10 \text{ e}/\text{\AA}^2$. The iDPC-STEM images were acquired with the total exposure dose of $10 \text{ e}/\text{\AA}^2$ and two convergence semi-angles 6.43 mrad (middle) and 2.58 mrad (right), respectively. The pixel size of iDPC-STEM imaging is 1.1 nm. The original pixel size of TEM images was 1.05 nm and then resized to 1.1 nm by FFT cropping algorithm. (d) Quantitative comparison of contrast between TEM and iDPC-STEM images for different thickness of sections. The contrast of each micrograph was measured using

1 the line profile method (see **Figure S3**). For each data point, the measurement was
2 repeated three times using different profile lines at different regions.

3 **Figure 5. Depth-sectioning using iDPC-STEM imaging for a thick section.** The
4 resin embedded mouse testis tissue was prepared using Method 1 and sectioned to
5 thickness of 600 nm. Series of iDPC-STEM images were acquired with convergence
6 semi-angle of 6.43 mrad and the exposure dose of 3 e/Å². The pixel size was set to 5
7 nm. The defocus step between selected images is 50 nm. The central slices covering
8 almost the full thickness of the sample area are shown sequentially from top to bottom
9 with the focus positions centered in the middle of the sample.

10

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Theoretical vertical cross section of the probe along the direction of 200keV electron beam propagation (from top to bottom) with different convergence semi-angles. Top row, probe corrected system ($C_s=0$). Bottom row, probe non-corrected system ($C_s = 2.7$ mm). For the non-corrected system, the critical semi-angle at 7.4 mrad where the probe reaches the smallest size. The cross section was straightforwardly generated using Fresnel propagator (or equivalently defocusing) starting from the focus position of the condenser system (Kirkland, 2010).

Figure S2. Sample preparation procedures of Method 1 and Method 2 (see also Materials and Methods).

Figure S3. The contrast measurement by using line profile method. Step 1, the images normalization according to the pixel variance. Step 2, the line profile generation at the region of interest. Step 3, the contrast computation as difference between maximum and minimum intensity along the profile, normalized with the maximum.

Figure S4. Comparison between TEM and iDPC-STEM imaging at different electron doses for another section of HepG2 cell. (a) TEM and iDPC-STEM micrographs acquired with the total electron doses of $1 \text{ e}/\text{\AA}^2$, $3 \text{ e}/\text{\AA}^2$, $6 \text{ e}/\text{\AA}^2$, $10 \text{ e}/\text{\AA}^2$, $20 \text{ e}/\text{\AA}^2$ and $40 \text{ e}/\text{\AA}^2$, respectively. The pixel size TEM imaging is 1.04 nm. The pixel size of iDPC-STEM images was 1.1 nm. The convergence semi-angle of iDPC-STEM is 6.43 mrad. (b) Quantitative comparison of contrast between TEM and iDPC-STEM images at different exposure doses. The contrast of each micrograph measured using the line profile method (see **Figure S3**). For each data point, the measurement was repeated three times using different profile lines at different regions.

Figure S5. Comparison between TEM and iDPC-STEM imaging for different amount of staining using another section of HepG2 cell. (a) The sections prepared using Method 1 and Method 2, respectively. The thickness of the sections is 70 nm. TEM images were acquired with the defocus of $\sim 7 \text{ }\mu\text{m}$ and the total exposure dose of $10 \text{ e}/\text{\AA}^2$. The iDPC-STEM images were acquired with the total exposure dose of $10 \text{ e}/\text{\AA}^2$

1 and two convergence semi-angles 6.43 mrad and 2.58 mrad, respectively. The pixel size
2 of TEM imaging is 1.04 nm. The pixel size of iDPC-STEM images was 1.1 nm. **(b)**
3 Quantitative comparison of contrast between TEM and iDPC-STEM images for
4 different stained sections. The contrast of each micrograph measured using the line
5 profile method (see **Figure S3**). For each data point, the measurement was repeated
6 three times using different profile lines at different regions.

7 **Figure S6. Comparison between TEM and iDPC-STEM imaging upon different**
8 **section thicknesses using another testis tissue specimen. (a)** The sections were
9 prepared using Method 1 with different thickness of 200 nm, 400 nm and 600 nm,
10 respectively. TEM images (left) were acquired with the defocus of $\sim 7 \mu\text{m}$ and the total
11 exposure dose of $10 \text{ e}/\text{\AA}^2$. The iDPC-STEM images were acquired with the total
12 exposure dose of $10 \text{ e}/\text{\AA}^2$ and two convergence semi-angles 6.43 mrad (middle) and
13 2.58 mrad (right), respectively. The pixel size of iDPC-STEM imaging is 1.1 nm. The
14 pixel size of TEM images was 1.04 nm. **(d)** Quantitative comparison of contrast
15 between TEM and iDPC-STEM images for different section thicknesses. The contrast
16 of each micrograph measured using the line profile method (see **Figure S3**). For each
17 data point, the measurement was repeated three times using different profile lines at
18 different regions.

19 **Figure S7. Noise reduction effect by integration step in iDPC-STEM. (a)** Noise
20 propagation into iDPC-STEM image. Starting from hypothetical, ideal ($\text{CTF}=1$),
21 noiseless iDPC-STEM image, the two gradient components (differentials, DPC_x and
22 DPC_y) are formed (ideal, noiseless DPC-STEM images). In practice, the two DPC-
23 STEM components acquired directly by measurement are always noisy. Therefore,
24 Gaussian noise is added and inverted gradient operator (integration) is applied (Lazić
25 et al., 2016) to form the noisy iDPC-STEM image. **(b)** Comparison of spectrum signal-
26 to-noise ratio (SSNR, red curves) between obtained noisy DPC-STEM (x and y
27 direction) image and final iDPC-STEM image. Because ideal noiseless images are
28 available, the SSNR is computed directly by dividing the spectrum of the noiseless
29 image (signal) with the spectrum of the difference (noise) between the noisy image and

1 the noiseless image. The spectra of the noiseless images are shown for reference (blue
2 curves), each normalized to corresponding maximum value. As we can see, even though
3 amount of noise added to DPCx/DPCy is such to produce SSNR of 2.7 at the maximum
4 spectral component, the resulting SSNR for iDPC-STEM image is very close to 4 at the
5 same frequency. Clearly, iDPC-STEM reduces noise and increases SSNR. **(c) (d) (e)**
6 More comparison of SSNR between DPC-STEM and iDPC-STEM images with
7 increasing noise levels. The Gaussian noise added to DPC-STEM to result in SSNR at
8 the spectral maximum reaching (c) 4, (d) 2.7 and (e) 1.4, respectively. Corresponding
9 iDPC-STEM maximum SSNR reached (c) 5, (d) 4 and (e) 2.7, respectively. Left (c-e):
10 the DPCx-STEM component without/with Gaussian noise (top) and the corresponding
11 SSNR curve (bottom). Right (c-e): the corresponding noise-free and obtained noisy
12 iDPC-STEM image (top) and the corresponding SSNR (bottom).

13 **The two main crucial observations: (1)** The SSNR ratio between iDPC-STEM and
14 DPC-STEM increases with more noise added (at maximum (c) 5/4 (d) 4/2.7 and (e)
15 2.7/1.4). **(2)** Most of the iDPC-STEM SSNR spectrum stays above 1 in all the cases.

16

Table 1. The dependency of STEM resolution and depth of focus with the convergence semi-angle for the 200 keV electron (wavelength $\lambda = 2.508 \text{ pm}$)*.

Convergence semi-angle α [mrad]	3 pixel resolution at proper pixel size [Å]	Ultimate resolution $\lambda/(2\alpha)$ [Å]	Proper pixel size for ultimate resolution (Nyquist criterion) [Å]	Depth of focus $2\lambda/\alpha^2$ [nm]
1	18.7	12.5	6.2	5016
2	9.4	6.3	3.1	1254
3	6.3	4.2	2.1	555
4	4.6	3.1	1.5	312
5	3.7	2.5	1.2	200
6	3.1	2.1	1.0	139
7	2.7	1.8	0.9	102

* Column 3 shows the ultimate resolution allowed by convergent semi-angle defined by condenser aperture. This is true for any STEM (except Ptychography, which uses iterative reconstruction schemes (Muller D. (2019))) and is derived from physics of the STEM imaging formation process. This resolution is obtainable assuming small pixel size (scanning step) and infinite electron dose (infinite SNR). In practice, this can be achieved using relatively small ($\sim 50 \text{ nm}$) fields of view (FOW) and relatively large image sizes ($\sim 1\text{k} \times 1\text{k}$ number of pixels), producing acceptably small pixel sizes ($\sim 20 \text{ pm}$). At the same time, with the high beam currents ($\sim 50 \text{ pA}$) and/or longer dwell times ($\sim 10 \text{ }\mu\text{s}$), large electron doses ($\sim 100\,000 \text{ e}/\text{\AA}^2$) are easily produced. To examine biological features of interest in their full-size, investigations of a relatively large FOW areas ($\sim 1 \text{ }\mu\text{m}$, often larger) are required. Therefore, even with $4\text{k} \times 4\text{k}$ image sizes applied, the smallest available pixel sizes become relatively large ($\sim 2 \text{ \AA}$, often larger). Also, minimal electron doses of the beam, less than $50 \text{ e}/\text{\AA}^2$, are allowed in order not to significantly damage the sample. This limits the beam currents down to order of magnitude lower values ($\sim 5 \text{ pA}$) as well as the dwell times ($\sim 1 \text{ }\mu\text{s}$).

Table 2. A summary of comparison between TEM and iDPC-STEM imaging in different experiments.

	TEM ¹	iDPC-STEM
Contrast of samples in varies dose: Total dose < 10 e/ Å ² Total dose > 10 e/ Å ²	Medium High	High Very high
Contrast of samples with varies amount of stain: Method 1 (normal OsO4 + post stain) Method 2 (normal OsO4)	High Medium at membrane area but weak at protein area	High High at both membrane and protein area
Contrast of samples with varies thickness: 200nm 400nm 600nm	High Medium Weak	High High High ²
Depth sectioning	Not available	Possible ³

3

4 ¹ all TEM images were taken on FEI Talos F200C with an FEI Ceta camera.

5 ² convergence semi-angles of 2.58 mrad

6 ³ convergence semi-angles of 6.43 mrad

7

Figure 1

a

b

Figure 2

Figure 3

Figure 4

Figure 5

Figure S1

Figure S2

Figure S3

Step1: Normalization

$$I_{norm} = \frac{I - I_{min}}{I_{max} - I_{min}}$$

Step2: Line profile of interested area

Step3: Contrast calculation

$$Contrast = \frac{I_{max} - I_{min}}{I_{max}}$$

Figure S4

Figure S5

Figure S6

Figure S7

a

b

c

d

e