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Recent Developments in Direct Electron Detectors for Electron Cryo-Microscopy

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A number of technical improvements in the design of backthinned CMOS detectors for electron cryo-microscopy are reviewed in this paper. The resulting improved performance, in terms of DQE at all spatial frequencies, has enabled structural investigations to be carried out at higher resolutions than previously possible with film or phosphor-fiber-optic-coupled CCD detectors – this is illustrated with a few chosen examples.

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1. Introduction

A primary goal of structural biology is to elucidate the structure of large biological macromolecules to atomic resolution, which leads to an improved understanding of how they function and can assist in therapeutic drug design. The traditional route to high resolution structure determination, when good three dimensional crystals are available, is X-ray crystallography. However, there is a large class of molecules, such as membrane proteins, which are difficult or impossible to crystallize for which electron cryo-microscopy (cryo-EM) of isolated individual particles provides an excellent alternative. Cryo-EM is increasingly being applied as a complementary tool with crystallography, for example when it is important to investigate modifications in a structure.

Over the past decade we have used a number of pixel detectors, such as hybrid and CMOS detectors, in an attempt to replace the conventional data recording medium, namely film [1] with a more convenient detector. Recently, following several cycles of incremental improvements, we have developed backthinned CMOS detectors, which have superior performance to all previous categories of detectors, including film. It was clear however, from initial tests conducted in our laboratory [2] and elsewhere [3] that several problems would need to be solved to achieve a detector that could satisfy the needs of cryo-EM. Improvements required included : considerable radiation hardening to prolong the lifetime of the detector in the microscope to reach ~ a few years in operation[4], larger sensitive areas, larger number of pixels, backthinning to <50 microns to reduce backscatter and to improve DQE at all spatial frequencies[5], faster readout for recording images in movie mode (more below) and investigating the option of electron counting mode for obtaining close to near-perfect DQE at high spatial frequencies [6]. Since biological specimens have very low contrast and the electron dose is limited due to radiation damage the images are very noisy with poor signal-to-noise ratio. It is imperative therefore to use detectors, with very high efficiency and count every electron without adding extra noise (high DQE). A recent review has a useful comparison of the performance of number of direct and indirect electron detectors for a range of energies[7].

Some examples from current projects will be used for illustrating the unique benefits of CMOS detectors for high resolution cryo-EM. In particular, we will show how close we can get to atomic resolution and which further improvements are needed to the present detector for even better performance.

2. Single Particle Analysis

Since cryogenic preservation was introduced for biological specimen to reduce radiation damage many groups have been working to obtain higher resolution structures. There are many cases where, due to the charge distribution on the molecule or other reasons it is not possible to obtain even 2D crystals and so single particle analysis, which consists of studying the structure of isolated macromolecules, is becoming very popular. An important requirement for this technique is that macromolecules need to be purified into a near-homogenous sample but this is a less riorous requirement than the amount of preparation needed to make crystals. Obtaining high resolution structures from single particle analysis is more difficult than electron

crystallography on 2D crystals since, unlike 2D crystals, the molecules are imaged in random orientations and the alignment prior to averaging all the single particles selected from a large number of images has to be performed by specialised software. As the radiation damage limits the signal that can be obtained from individual particles there are considerably greater demands on the detector performance in terms of DQE (both at zero and high frequencies). Further details on single particle analysis can be found in some recent publications [8-10].

3 CMOS Sensors for electron microscopy

As mentioned earlier, film played a historic role as the standard detector employed in high resolution imaging. The quest to replace film with an electronic detector was motivated by the need for a more convenient detector, which did not involve several tedious intermediate steps before data was accessible. Several indirect (phosphor-fibre-optic-coupled CCDs) and direct(hybrid and monolithic active pixel sensors(MAPS)) detectors were evaluated for their suitability for recording electron microscope data [1]. A comparison of the properties of the detectors, namely film, CCDs and CMOS in terms of the modulation transfer function (MTF) and detective quantum efficiency (DQE) as a function of spatial frequency at 300 keV, is shown in Fig.1; results for hybrid detectors, which were carried out only at 120 keV are not included in this figure. The CMOS detector showed very promising results compared with the other two detectors, namely film and the other electronic detector, CCD. However, the DQE, though comparable to film at Nyquist frequency was not as good as film at ~0.5 Nyquist frequency [11].

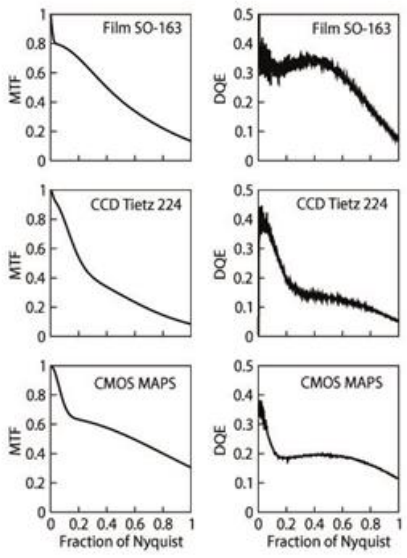

Fig.1 Comparison of film, CCD and non-backthinned CMOS detectors in terms of MTF and DQE as a function of spatial frequency at 300 keV [11].

In an attempt to understand the reasons for the low DQE, Monte Carlo simulations on the behaviour of electrons in silicon were carried out. The simulations were carried out with 300 keV electrons incident normally on a 350 µm thick detector made of silicon with a 35 µm front layer as shown in Fig.2 [5]. The thin grey layer represents the thickness likely to be obtained for a backthinned detector. The epilayer in which the signal is generated is only a fraction of this thickness, 5-15 µm, at the top edge of the figure. The majority of electrons go straight through the epilayer and are stopped in the 'substrate' after suffering multiple scattering. The red portion of the trajectories show the section of the electron tracks which lead to energy deposition in a backthinned detector; the black tracks also lose energy but, as it is in the substrate, do not contribute to the signal. A small fraction of the electrons are backscattered, shown as white tracks, which do contribute to the signal but in an undesirable fashion: they produce false positional information, lowering the measured spatial resolution or MTF. However, the white tracks would be removed in a backthinned detector. As the backscattered signal is due to lower energy electrons it is generally higher than the signal from the 'primary' electrons, which initially traversed the top surface (red lines). The additional white tracks would contribute a low-resolution component to the signal together with contributions to the noise at both low and high spatial frequencies. By observing the signals due to single electrons, at extremely low dose rate (1electron/5000 pixels/second), when two electrons in the observed area are extremely unlikely, two signals are often observed in close proximity with one signal much higher than the other suggesting the correctness of the assumption.

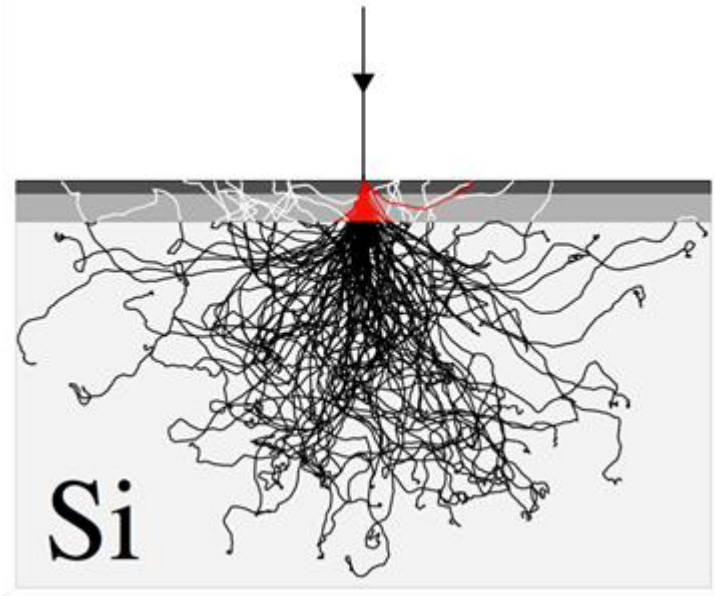

Fig.2 Monte Carlo simulation of the expected trajectories of 300 keV electrons in silicon. The signal generating epilayer region, typically ~ 10 µm, is shown as dark grey and the 'expected' backthinned sensor sensor in light grey. Backscattered electron tracks, which reduce MTF and DQE are shown in white[5].

To test this in practice, a comparison was made between the MTFs obtained with three sensors of varying thickness:700µm, 50µm and 35µm and shown in Fig.3. As expected, the

MTF is higher for the backthinned sensor and the improvement is particularly noticeable at higher frequencies. Since the DQE is proportional to MTF^2 it also benefits from an enhanced MTF. In addition to reducing backscattering within the sensor, scattering from the support structures immediately below the sensor is also important and should be reduced as much as possible [11]. The experimentally measured DQE as a function of spatial frequency for thin and thick epilayers is compared with that of an ideal detector in Fig.4.

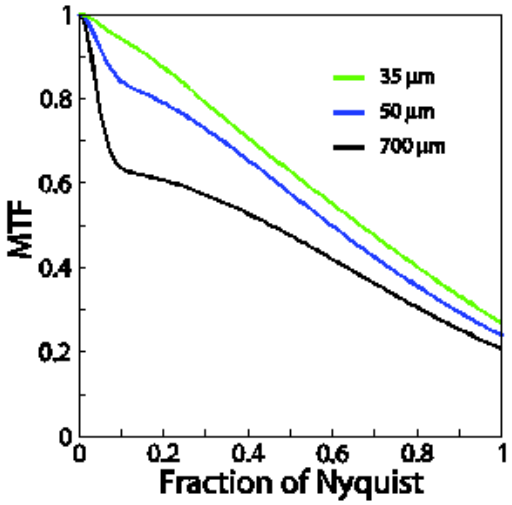

Fig.3 Improvement in MTF as a function of spatial frequency for 300 keV electrons from different amounts of backthinning [5]. The thinnest sensor, with 35 µm thickness has the highest MTF.

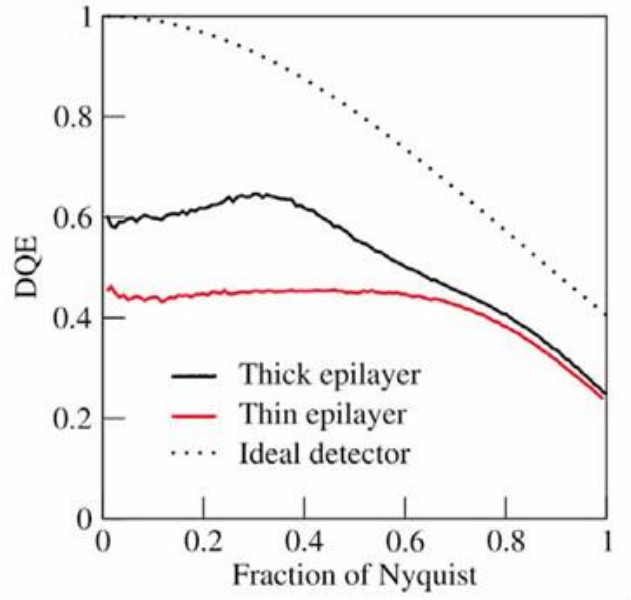

Fig.4 DQE as function of spatial frequency for thin(red line) and thick epilayer(black line) sensors and comparison with an ideal, pixellated detector, shown as a dotted line.

Further significant improvements in MTF and DQE as a function of spatial frequency were achieved by recording (or counting) individual electrons and obtaining their impact location to sub-pixel accuracy, rather than using the conventional analog method used for CMOS readouts.

The counting method relies on reducing the number of electrons incident during each frame so that images of individual incident electrons are recorded. In practice this requires very high frame rates as the alternative of reducing the incident dose rate results in exposures that are too long and so susceptible to external drift. The single electron images can be processed in many ways [6, 12]. An increase in the low spatial frequency DQE is obtained by simply counting each electron with the same weight and so eliminating the intrinsic variability associated with energy deposited by incident electrons. Obtaining improved DQE at higher frequencies is more difficult and relies on accurately determining incident electrons position from the recorded image. This is usually done by some form of centroiding but for the detector and incident electron energy used in [6] this method failed to give an improved DQE towards the Nyquist frequency. In this case the accuracy with which the incident electron positions could be determined, and hence MTF enhancement, did not outweigh the increase in the noise power spectrum at higher frequencies resulting from localising incident electrons. An approach in which the images of the events were treated as a probability distribution for the electrons incident position did however lead to an improved DQE over all spatial frequencies as shown in Fig.5.

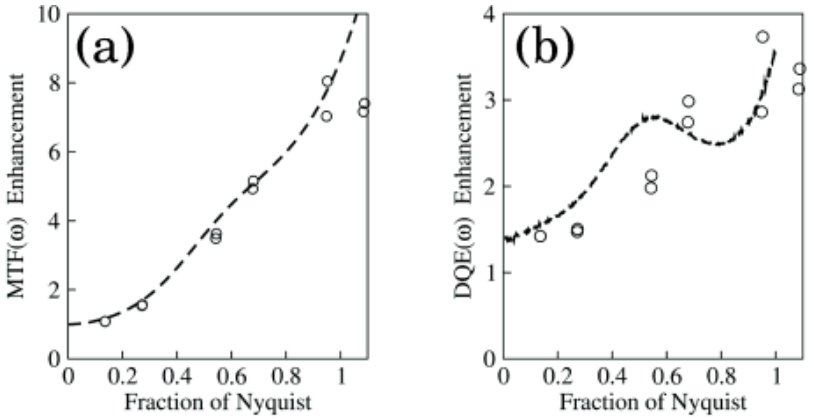

Fig.5 Experimental observation of improvements in MTF and DQE as a function spatial frequency obtained by electron counting compared with conventional analog readout methods[6].

4. How can CMOS detectors help obtain better images?

A major limitation in obtaining high resolution images is that biological specimens are susceptible to radiation damage and the total dose that can be used before finer features are destroyed is low, typically ~20 electrons/Å^2. What makes imaging biological specimen challenging is that the contrast for the specimens, embedded in vitreous ice for cryo-

microscopy, is very poor. However it is not possible to increase the signal-to-noise ratio by increasing the electron dose due to radiation damage.

Another major cause of poor resolution that has long been recognized is movement of the sample during imaging causing blurring [13]. It was thought that the movement was a result of beam-specimen interaction and that it could be eliminated or at least reduced by minimising the dose. One of the applications of the noiseless Medipix2 detector [14] was to measure the effects of ultra-low dose electron beam on specimen movement: experimentally, the dose rate was reduced to 500 electrons/frame (0.003 electrons/ $Å^2$), which is several orders of magnitude lower than the dose rate used in regular cryo-imaging. This protocol was made possible by the detector being essentially noiseless [15]. The low dose was achieved by recording the exposure as a movie with 400 frames each frame containing very few electrons. Somewhat surprisingly, even at these ultra-low doses, the images were still blurred, suggesting that the amount of specimen movement was dose dependant rather than dose-rate dependant. It had been suggested [13] that stroboscopic imaging, i.e. movie mode imaging, would be useful in obtaining higher resolution because the movie frames could be aligned against one another later, thus deblurring the image. By reducing the electron dose in each sub-exposure the movement of the specimen would be correspondingly reduced. By adding all the sub-exposures it would then be possible to arrive at a higher resolution. The first attempt to remove blurring by recording a series of images was made using a negatively stained virus molecule with Medipix2[16]. Fast readout CMOS detectors, used in the 'movie' mode allow these ideas to be tried in practice in cryo-EM. The reason it has taken so long for these experiments is that detectors combining high DQE and fast readout have only recently become available [17] - as illustrated by some examples in the following section.

5. Applications of Direct Electron Detectors

The first of three CMOS direct detectors available commercially, are considered briefly in this review. The DE-12 fast readout direct electron detector was used in a comparison with a CCD detector under very similar conditions and was found to have a superior performance[18]. Image blurring, caused by the movement of molecules due to beam interaction with the specimen, as discussed above [13], was studied by Brilot, et al [19] using the DE-12. The single particles chosen for this study, the rotavirus double layer particles (DLP), have a very regular structure with a molecular weight of 70 MDa [20]. Images, collected in a 'movie-mode' containing 40 frames/second, were analysed for the amount and direction of motion of the virus particles during the exposure. The authors showed that the ice layer, in which the virus particles are embedded, was moving in a drum like fashion with particle rotations of a few degrees and translational movements of ~10Å. These motions have a greater effect on the high resolution features of the structure, and may be the most significant cause of blurring in single particle images. An interesting finding by the authors was that particle motion was greater during the early part of the exposure compared to the later frames. It was shown that by correcting for the particle motion it was possible to restore some high resolution features in the image, i.e. remove some of the blurring.

The second CMOS detector, the Falcon II was used in a new method for validating 3D cryoEM maps, described by Chen, et al [21]. The protocol was based on using a test structure,

namely beta-galactosidase at 6Å resolution obtained with the Falcon CMOS detector [22] and shown in Fig.6. The higher DQE of the backthinned Falcon II allows much higher resolution maps with fewer single particles compared to film. Film required 49000 single particles for obtaining 11 Å resolution whereas Falcon II needed only 43000 particles to obtain ~6Å resolution. Incidentally, beta-galactosidase with a molecular weight of 450 kDa is one of the smallest molecules to have its structure obtained at this resolution. The authors have recently improved the resolution to 4.0 Å (R.Henderson, personal communication).

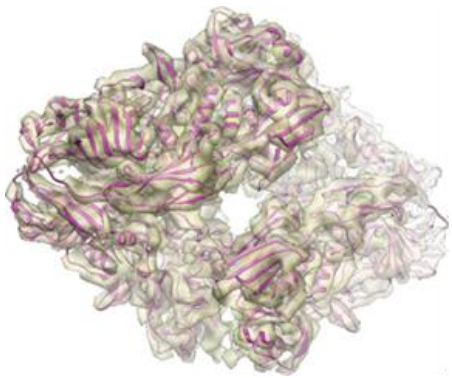

Fig.6 Medium (6Å) resolution map of beta galactosidase obtained by cryo-EM and comparison with model[21].

Scheres, et al have developed movie processing software based on a Bayesian approach, which they have applied very successfully to ribosome structural analysis using data collected on a backthinned Falcon II electron detector [10]; a prototype of the detector was described previously [17]. The software includes a new statistical movie-processing approach, which makes it possible to correct, at least partially, for any movements of the specimen during an exposure. The authors recorded 16-frame movies during a 1 second exposure, each frame containing < 1 electron/Å^2 – clearly, the very low noise of the detector is crucial for the success of this project. As mentioned earlier, in order to obtain accurate high resolution features of the structure it is essential to align all molecules as precisely as possible. This process is difficult as the images of individual molecules are noisy and the software needs to distinguish between signal arising from the molecule and inherent noise in the image.

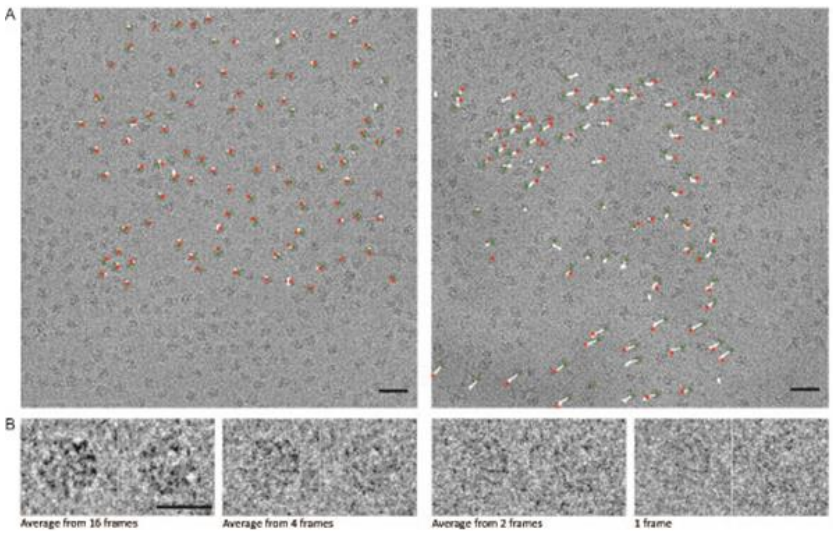

Fig.7 70S ribosome data collected on Falcon II. Data averaged from two 16-frame videos shown in image; scale bar 500 Å. First four frame averaged particles shown as green circles and last four frames in red circles – connected with white lines. Difference in positions exaggerated by 25 times for clarity. Lower figure illustrates decreasing SNR with number of averaged particles [10]. Scale bar: 200Å.

The superior S/N properties of the Falcon II enabled the alignment of the same molecule (ribosome) was lower, 2° compared with 4° obtained with phosphor-fibre-optic-coupled CCD detectors in previous experiments[23]. This is a huge improvement in single particle reconstruction and has led to a dramatic improvement in the structural analysis. Higher S/N means that fewer particles are required for averaging to obtain the same accuracy. Better alignment also permits higher resolution structures to be obtained with fewer particles. Applying the statistical movie processing software, this allows a resolution of ~4Å from ~35,000 particles. Further improvements to the processing programs have yielded a further improvement in resolution to 3.6 Å (Scheres, Personal Communication). The previous best resolution with film as recording medium was obtained by averaging 1.4 million ribosome particles (i.e. about two orders of magnitude more) to result in 5.5 Å resolution [23].

Finally, using a fast readout and high DQE (Gatan) counting detector, Li, et al [10, 18] have been able to obtain near-atomic resolution maps of the 20S proteasome, a 700 kDa protein with D7 symmetry [24]. The detector has 3840 x 3712 5 µm pixels and the fast readout allows 40 full frames/second [25] which makes movie-mode possible. They were able to correct the beam-induced movements to sub-pixel accuracy. They also studied the effect of dose rate, which may result in coincidence loss at higher rates, on the signal-to-noise ratio[24]. Their main conclusion was that there was little effect on the SNR; in particular the high frequency signals were unaffected.

6. **Conclusions**

We have presented a brief overview of the the backthinned CMOS electron detectors, which have made a dramatic impact oncryo-EM. The detectors has surpassed the performance, in terms of DQE as a function of spatial frequency, of film and phosphor-fibre-optics-coupled CCD detectors. The higher DQE allows improved three dimensional models, which in turn allows better orientations of the molecules in the image to be determined resulting in an improvement of three dimensional models. As a result near-atomic models can now be obtained with fewer particles. Due to the fast readout it is also possible to collect data in ‘movie-mode’, considerably reducing the image blurring artefacts due to beam-specimen interactions. The new and powerful tool is allowing many cryo-EM groups to obtain structural information previously restricted to X-ray crystallography and we have included only presented a small sample of high resolution macro-molecular structures from recent literature.

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