Some personal and historical notes on the utility of "deep-etch" electron microscopy for making cell structure/function correlations

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ABSTRACT **This brief essay talks up the advantages of metal replicas for electron microscopy and explains why they are still the best way to image frozen cells in the electron microscope. Then it explains our approach to freezing, namely the Van Harreveld trick of "slamming" living cells onto a supercold block of metal sprayed with liquid helium at –269°C, and further talks up this slamming over the alternative of high-pressure freezing, which is much trickier but enjoys greater favor at the moment. This leads me to bemoan the fact that there are not more young investigators today who want to get their hands on electron microscopes and use our approach to get the most "true to life" views of cells out of them with a minimum of hassle. Finally, it ends with a few perspectives on my own career and concludes that, personally, I'm permanently stuck with the view of the "founding fathers" that cell ultrastructure will ultimately display and explain all of cell function, or as Palade said in his Nobel lecture,electron micrographs are "irresistible and half transparent … their meaning buried under only a few years of work," and "reasonable working hypotheses are already suggested by the ultrastructural organization itself."**

After hyping "deep-etch" electron microscopy (EM) for my whole career (Heuser, 2011), I'll take this invitation to write an ASCB award essay to talk it up some more! Some will say that this is "flogging a dead horse," but I really think not. The advantages of metal replicas for EM are just too *huge*. Replicas are not only impervious to beam damage in the electron microscope, forever the big problem, because the electron beam heats up the sample so terribly during viewing, but their electron-scattering power is also excellent, so they are simple to image and give super high-contrast. And the key thing to remember is that replicas are utterly faithful to whatever they are replicating—they're just surface renderings, copying exactly the contours of the sample and displaying these contours in the electron microscope image. So the whole approach boils down to worrying about how to prepare your biological samples for replication. (Well, I can't claim it's quite that simple. It takes the right equipment and some practice to make a proper replica, but, once mastered, it's utterly routine and simple to learn. When Mark Kirschner first watched me do it—while helping me to put it on the map by providing gorgeous cytoskeletons [Heuser and Kirschner, 1980]—he got bored right away and asked me, "Can't you teach a monkey to do that?")

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Anyway, replicas have a glorious history, because in the early days of EM, way before thin-sectioning techniques had been developed, they were the only way to go—the only way to get any sort of biological sample into the electron microscope. Thus the EM pioneers in the 1940s used metal replicas to discover viruses and phages and to make the first halting characterizations of macromolecular assemblies like collagen and neurofilaments. What they lacked back then was a way to see inside cells, which

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Abbreviations used: EM, electron microscopy; SEM, scanning electron microscopy.

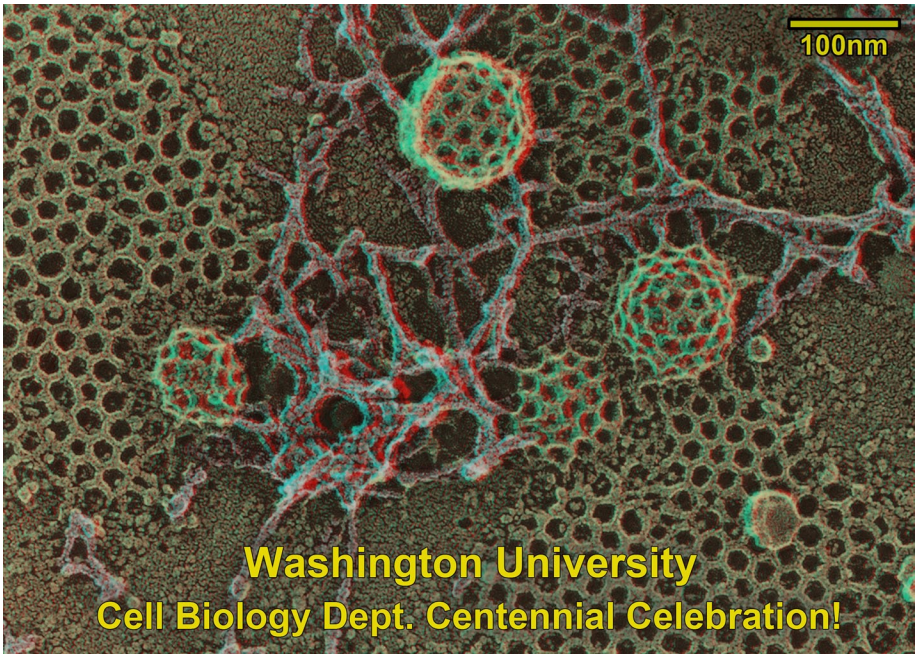

FIGURE 1: A platinum replica of the inside surface of a HeLa cell prepared by "unroofing" it in culture before quick-freezing and freeze-drying it in the usual way (Heuser, 2000). This fun "anaglyph" three-dimensional view was used for the publicity and table cards for our department's centennial celebration three years ago. It focuses on the various "honeycomb" clathrin lattices found on all cell membranes and illustrates the various stages in their evolution, from totally flat to fully curved and ready to pinch off during endocytosis. Such three-dimensional deep-etch images were the first to illustrate that F-actin filaments (highlighted in purple) often become involved in the later stages of such clathrin coated–pit formation and stay behind as circular "scars" after coated vesicles have left the surface (above the "Wash" in Washington University). As explained in this essay, the swell opportunity to view such expanses of the plasma membrane at such a high resolution was a lucky outcome of our being able to freeze samples fast enough to avoid ice-crystal formation and then, miraculously, to platinum-replicate such frozen membranes without melting them.

Keith Porter achieved for the first time in 1945 by simply growing cells flat enough to see through in the electron microscope—really, really flat—and then fixing and staining them properly for EM (his other huge contribution). People not familiar with EM should be reminded that Porter's 1945 images opened the door to cell biology, and his development of thin-sectioning techniques for cells in the following 10 years really put cell biology on the map.

But back to replicas. The whole field of scanning electron microscopy (SEM) was totally dependent on them because everything had to be coated with metal in order to be seen in the scanning electron microscope. Likewise, the exciting field of freeze-fracture EM took off after Hans Moor teamed up with a Swiss company that made replicating machines (Balzers of Lichtenstein) and mounted a microtome inside one, so that frozen cells could be fractured open (not quite thin-sectioned, the microtome wasn't that good). This made it possible for people to make metal replicas of frozen cells without melting them even a little bit—some sort of miracle!

Deep-etch EM is a variant of what Moor introduced (Heuser and Salpeter, 1979) and deserves special attention only because its purpose has been to avoid all of the fixation and staining and dehydrating procedures that had accompanied previous approaches to EM and essentially to get living cells replicated after they were frozen (Figure 1). We found that freeze fracture works just as well or better on unfixed cells and molecules, and therefore made a huge effort to devise a really good way to freeze living cells, tissues, and cell extracts without introducing such artifacts as ice-crystal damage.

The best way to freeze everything turned out to be a spruced-up version of an approach Anthonie Van Harreveld had used in the 1960s at CalTech to freeze brains in preparation for classical thin-section EM. Van Harreveld wanted to maintain the proper distribution of electrolytes in the brain and had reason to believe that the classical fixation techniques being used on brain were distorting this distribution. He reasoned that the "freeze-substitution" technique that Ned Feder and Richard Sidman had put on the map in the late 1950s would give him more realistic views. With this technique, a frozen sample is fixed and prepared for embedding in plastic by dissolving the ice out of it at subzero temperatures, using acetone or the like. Van reasoned, quite correctly, that this should prevent artifacts from occurring during fixation, because nothing ever melted; but how he came up with the idea to freeze the brain by "slamming" it onto an ultracold block of copper remains a mystery to this day. (It's fun to mention here that Van Harreveld didn't start developing this technique until he was already 60 years old!)

Anyway, it sure worked for Van, and it also worked for Tom Reese and me when we copied his "slammer," even though we had to spend years ironing out the bugs and making a freezing machine that was mechanically sound and gave reproducible results (Heuser *et al.*, 1979). The result was our so-called liquid helium–cooled "cryopress" (renamed to avoid the distressing idea of a delicate piece of tissue being "slammed" against anything—albeit, it's the abruptness of contact and the superfast extraction of heat from the sample by the copper block that gives such good freezing in the first place). Fast-forward to today, and we find that freeze substitution is *still* the backbone of modern efforts to image cells in the electron microscope, and indeed preserves the structure of cells far better than the techniques of fixation and plastic embedding developed by the pioneers of thin-section EM. When combined with thicker sections, higher EM voltages, and modern tomographic reconstruction techniques, it yields really outstanding images.

So why aren't there more than 10 labs in the world using our (or Van Harreveld's) cryopress to get the quality of freezing our lab has depended on for *decades*? The answer lies in part with another advance that Hans Moor spearheaded in Switzerland, again with the same enlightened Balzers company producing vacuum evaporators, namely, high-pressure freezing. At the time, phase diagrams of water indicated that water could be frozen into an amorphous glass without the induction of any damaging ice-crystal formation by putting it under extreme pressure (>2000 atm). Today, theories about how water turns into vitreous (noncrystalline) ice are much more complex, but Moor went ahead and developed ways to put a biological sample under huge pressures and only then freeze it by spraying liquid nitrogen at it rather than slamming it against a liquid nitrogen–cooled copper block. (The rapidity of freezing, he reasoned, should no longer be important if the pressure trick works—as apparently it does.) Today, most EM labs have a high-pressure

freezer, and most of the EM papers that are published on freeze-substituted cells have availed themselves of these devices.

So why not use our "slammer" (or cryopress) for freezing before freeze substitution, since it's cheaper, faster, more reliable, and handles larger samples? Frankly, we don't get it! Not only that, but high-pressure frozen samples cannot be freeze-fractured at all—at least no one has yet devised a way to do so—because the samples end up encased in various sorts of metal pressure chambers, whereas our quick-frozen or "cryopressed" samples are spread out and open to the world (mandatory for freeze fracture, but also good for freeze substitution). And for that matter, why aren't more labs making good old replicas of quick-frozen, deep-etched molecules (Heuser, 1983; Goodenough and Heuser, 1984; Hanson *et al.*, 1997)? That is, of course, the ultimate mystery to us. Probably it's just because people don't realize that there are still good replicating machines available for purchase, and people don't realize that these machines aren't so expensive and are easy to operate.

Well, as I said at the outset, I've been hyping our technique for decades and can't stop now. I believe that an opportunity is being missed and that simplifying techniques so that "even a monkey could do it" will attract not *monkeys* to the field, but serious young investigators who want to get their hands on electron microscopes and want to get the most "true to life" views of cells out of them with a minimum of hassle.

I'll close with some brief perspectives on my own career. I'm a photographer at heart and love sharing images, all sorts of images, with people who appreciate them and can learn from them—I love that more than anything. What fun it was, to be able to interact on a daily basis with the Mark Kirschners, Tom Pollards, Ron Vales, Bernie Gilulas, and Ira Mellmans of cell biology (and sorry to all those whom I didn't mention—you know who you are!). *Plus*, a handful of people really fired me up: Tom Reese, my boss as a postdoc at the National Institutes of Health, with whom I became so intertwined for so many years that he and I will never know who did what or who deserves what credit in the original development of quick-freeze, deep-etch EM (Heuser and Reese, 1973; Heuser *et al.*, 1979); and then Nobutaka Hirokawa, who came to my lab as a postdoc, and immediately orchestrated a host of collaborations with leading cell biologists around the world that put "deep etching" on the map (before leaving for the University of Tokyo to become chairman of the Department of Cell Biology, and then dean of the Medical School, and now head of the whole Human Frontier Science Program); and finally, my ex Ursula Goodenough, who absorbed my images and simply took off, making huge advances in several fields, thanks to her deep grasp of all aspects of cell biology.

Finally, I'd like to simply add this: biological EM was terribly interesting for me in the early days, back when it first allowed people to zoom in on the structures that light microscopists had been studying for so long and show what they actually were—what they actually looked like—what their "fine structure" was. I used to wait with eager anticipation for each new issue of the *Journal of Cell Biology* to arrive in the mail and then would devote a whole evening (maybe with a glass of wine) to carefully examining every new electron micrograph published that month. But EM became even more captivating for me as people began more and more to systematically manipulate cells by physical and pharmacological (and eventually genetic) methods and then to look in the microscope to see how this altered the fine structures of their cells. This opened the door to true structure/function correlations—at least when the effects of these experimental manipulations of cell physiology and biochemistry were properly determined, along with the microscopy.

This era of EM was the most fun for me, personally, but as it happened, this heyday was cut short by an overwhelming urge in some quarters to improve the methods of EM, in an attempt to make the imaging of cells more "lifelike." This trend particularly captivated the equipment manufacturers and led to an "arms race" of microscope development that ended up making electron microscopes so very costly that only a few centers could support them anymore. The result was actually a curtailment of general, everyday EM as it had been practiced by individual investigators in command of their own microscopes and published every month in the *Journal of Cell Biology*. And as a consequence, over the past 15 years or so, EM has gradually been relegated to a service status, carried out largely by EM cores in most major institutions. Gone is the primacy and independence of those who once considered themselves true "electron microscopists," and gone also is the use of EM for all sorts of fun structure/function correlations.

And helping to eclipse the "routine" EM that I enjoyed so much have been all the tremendous advances in light microscopy, coupled with all the advances in digital camera recording of live-cell dynamics (not to mention the burgeoning field of superresolution light microscopy, crowned this year with the Nobel awards). These huge advances have captivated nearly everyone still interested in functional correlations of cell structure and have left traditional EM sort of out in the cold, an outcome I find most unfortunate. I feel strongly that seeing cell structures at the EM level still is the only way to fully grasp their molecular architecture, and that seeing changes in their molecular architecture at this level is the only way to truly understand their function.

I'm permanently stuck with the founding fathers' view that cell ultrastructure will ultimately display and explain all of cell function! George Palade was my greatest hero, and his fun explanation in his Nobel lecture of why he chose to study the pancreatic acinar cell is my favorite quote: "Perhaps the most important factor in this choice was the appeal of the amazing organization of the pancreatic acinar cell, whose cytoplasm is packed with stacked ER cisternae studded with ribosomes. Its pictures had for me the effect of the song of a mermaid: irresistible and half transparent. Its meaning seemed to be buried under only a few years of work, and reasonable working hypotheses were already suggested by the structural organization itself."

Irresistible and half transparent, indeed! Thanks, George. And thanks to all of you who cared to look at my images and all the institutions and funding agencies that made it possible for me to generate them!

P.S. AN APOLOGIA

Every picture I take, I already have an audience for it right as I take it. I already have someone "looking over my shoulder." I'm already showing it to them, telling them about it. (Of course, they're not actually there, they may be continents away, but I'm imagining them being there and already planning how I will get that picture to them and what I'll tell them about it as soon as it's in the computer.)

I'm not kidding: every single picture I take is like that. It's for showing to someone who immediately comes to mind as soon as that field pops into view in the electron microscope. "Oh, Pietro will love that huge neuromuscular junction; Fulvio will be amazed by that quality of membrane preservation in freeze-substituted yeast; Ursula will be psyched by that run of axonemal dynein; Tom will be impressed with such a clear view of actin branchpoints." Only rarely am I lucky enough to have someone actually sitting next to me and to be able to talk to him or her right then, person to person—maybe a new postdoc or a close collaborator who

really needs to look over my shoulder to see how his or her prep came out.

Anyway, I want each of my real or imaginary viewers to *like* that picture, to think it's a good picture—attractive, clear, understandable, useful, illuminating, that is, illuminating something about the subject (be it a personal portrait or a picture of a cell interior or a molecule). I want my audience's appreciation! My whole drive of focusing all my work on improving techniques of preparation for EM has come from wanting to take better pictures and get more of that appreciation.

Besides that, there's just that darn old *curiosity*: what does it actually look like, what does it look like *exactly*? How good a picture of it can I take? How good-looking can I make it (or him or her, with my personal portraits)? (Nic Spitzer once irritably dubbed the latter my "thin sections of life" as I was clicking away while canoeing with him down a rapids on the Allagash River, but not paddling.) Always on my mind is what's the most expressive or most characteristic or "attractive" attire or decoration I can outfit it (them) with? Osmium or platinum or gold … or furs and silks? Capturing that best picture will help me to get to know my subject better, to really see it for what it is. Even artifacts can be extremely beautiful and informative, if one knows how one got them and what they say about what the structure was, before it got "altered."

All these aspects of photography I can appreciate by myself, all alone, but never as much as when there is just one other person with me, with the same inclination and proclivity. Sharing, mutual appreciation, communion—that has been the whole name of the game for me in my research career. My advisor Don Fawcett, one of the great masters of EM of all times, told me when I graduated from medical school, "Don't become an electron microscopist, you'll become everybody's slave." Actually, I think I can say that it turned out just the opposite: everyone else turned out to be my audience, my source of appreciation and self-worth, my foils, my mentors, and, most important of all, my best source for interesting things to look at in the electron microscope!

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