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INVITED REVIEW

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Double fertilization: a personal view

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Abstract This year marks the 100th anniversary of the discovery of double fertilization by Nawaschin in St. Petersburg, Russia and, independently, Guignard in France. This discovery came at the end of a period of controversy about fertilization in angiosperms and ushered in a new period of intense research. Still, by 1950, there were many unanswered questions about double fertilization because of limitations of the light microscope. The introduction of the electron microscope stimulated new research and helped resolve some of the questions. My own research with the electron microscope and that of people who worked in my laboratory is recounted and some of the still unanswered questions raised.

Key words Advances in microscopy · Double fertilization · Guignard-Nawaschin · Personal account

The late 1800s was a spectacular time for science. Discoveries were being made in chemistry, physics and, especially, biology. It was a period of intense competition and rapid advancement. Nowhere was this felt more than in research on plant reproduction. By this time, the epic battles over the nature of the pollen tube and the role of the pollen tube in fertilization were long past. The initial observations of the great Italian microscopist Giovanni Amici had withstood the assaults of Matthias Schleiden, who thought the embryo developed from the end of the pollen tube. The beautiful research of Wilhelm Hofmeister confirmed Amici's initial observations and finally, in 1856, Schleiden retracted his earlier observation and concluded that Amici and Hofmeister were correct.

The research that led to these conclusions was a result of the extraordinary development of the light microscope and the techniques necessary to make observations with this instrument. When the compound microscope was invented in the late 1600s, it was an inferior instrument. Plaqued with chromatic and spherical aberrations, as well as low magnifications, the early microscopes had limited application for serious research. Then, in the middle of the 1800s, research on the nature of light and its interaction with glass led to an understanding of optics, which, in turn, led to the production of lenses that controlled both kinds of aberrations. The center for these developments was in Germany, and soon German microscopes were the best in the world. As the understanding of interactions involved in producing good lenses improved, other people became involved in microscope design and lens production. Very soon, it was realized that there were limitations built into the light microscope and that these limitations were based on the nature of light itself. By the 1890s, light microscopes were being built that were at the peak magnification and resolution possible, some ×1200.

This limitation was accepted by researchers and they turned their attention to what they could see. The problem now became one of finding ways to treat the tissue so observations could be made. Thick sections of material could not be reviewed because the light would not go through. Yet, if thin sections were cut there was not enough matter to interact with the light to present an image to the observer. And, it was difficult to cut tissue thin enough to get light through. These various problems were overcome with a series of developments.

The first was the invention and use of the microtome. This is simply a refined method of cutting thin sections. The concept is an old one, going back to the 1770s, but was finally perfected in the late 1800s. The next development was the idea of killing the tissue and infiltrating it with a matrix that would hold it firm while it was being sectioned on a microtome. These procedures were perfected in the 1870s and 1880s at the Zoological Station in Naples. The matrix used was paraffin and the procedure spread rapidly throughout the world. To get the paraffin into the tissue, the tissue must first be killed and dehydrated; these procedures were developed simultaneously. One of the most famous of the fixatives used on plant tissue was developed by a Russian, Sergus Nawas-

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chin. Having successfully developed procedures to fix and section the material, it was now necessary to stain it. The researchers had available a remarkable collection of stains because of the development of aniline dyes by the chemical industry, primarily in Germany. These wonderful dyes had marvelous names: safranin, aniline blue, fast green, Bismark brown, gold orange, hematoxylin and many, many others. A beautiful, colorful world opened before botanical researchers and they took advantage of it.

The stage was now set for advances in plant embryology, and they came, thick and fast. Hanstein (1870) published a detailed account of embryo development in *Capsella* and Strasburger (1879) described the development of the megagametophyte of *Polygonium*. He also (Strasburger 1884) observed the fusion of male and female gametes to form the zygote in *Monotropa hypopitys*. The nature of the endosperm, although not its origins, was studied by Hegelmaier (1885, 1886) and Treub enthusiastically threw himself into the study of chalazogamy (Treub 1891), a phenomenon he greatly overrated.

But the main question remained unanswered: what happened when the pollen tube arrived at the embryo sac? By the 1890s it was generally agreed that the pollen tube contained two sperm, or male gametes. It was also agreed that one of these sperm fused with the egg to form the zygote that developed into the embryo. But what about the endosperm? Where did it come from and what stimulated its almost frenzied development? The answer, that both sperm were involved in the phenomenon now known as “double fertilization”, came, fittingly, from two laboratories situated a continent apart. Sergius G. Nawaschin had an active laboratory in St. Petersburg, Russia. Well known as an active researcher and inventor of new microscopic procedures, he published the first report of double fertilization in the fall of 1898 (Nawaschen 1898). Working with *Lilium*, he described the two sperm fusing with the egg and the polar nuclei to give rise to the embryo and primary endosperm nucleus (Nawaschen 1898). Hard on his heels was Leon Guignard, working in France with *Fritillaria*, who published an independent account of double fertilization a few months later, in 1899 (Guignard 1899). Both were well-known and experienced researchers and there is no doubt that they had reached their conclusions separately. Indeed, it is amazing that the phenomenon was not discovered earlier. A number of years before Nawaschin’s paper, another Russian botanist, W. Arnoldi, made preparations that showed double fertilization, but he mistook the second sperm nucleus as a misplaced nucleus that had entered the embryo sac during sectioning (Maheshwari 1950).

After the discovery of double fertilization, the pace of plant embryology investigations quickened, so much so that by 1903 Coulter and Chamberlain were able to summarize the information now available in their famous *Morphology of angiosperms* (Coulter and Chamberlain 1903). Coulter and Chamberlain were both at the newly formed University of Chicago and they were to start a tradition in morphological research that lasted for decades. One of their major competitors was Karl Schnarf at the University of Vienna who published major works in 1929 and 1931 (Schnarf 1929, 1931). During this early period E.C.R. Sorieger of France, W.W. Finn in the Ukraine, as well as many others in France, Germany, Italy, Sweden and the United States, were active.

In 1950, Professor Maheshwari, for many years a preeminent researcher in plant embryology, published *An introduction to the embryology of angiosperms*. This book, which is a masterful summary of the research on plant embryology, covers the literature before the introduction of the electron microscope. In it are discussed pollen development, the development of the megagametophyte, double fertilization and embryo/endosperm development; all are described on the basis of what can be seen with the light microscope. But many questions were unanswered. The condition of the sperm, whether or not they are true cells, was not known. The relation of the pollen tube to the embryo sac was also still unclear, as were many other questions, and for a very good reason: the answers were just not available with observations made with the light microscope. Research had stagnated in many areas because it was impossible to see what needed to be seen.

A new approach was needed, a new way of looking at the old problems, and we now had it: the electron microscope. At the time, my own involvement with that wonderful, frustrating instrument was not very long. My involvement in the question of double fertilization was, in one way, much longer and goes back to my undergraduate days at the University of Chicago. Immediately at the end of World War II, I enrolled in the University of Chicago. I entered a fantastic university at an exciting period of time. I had developed an interest in plants in high school and naturally gravitated toward the Department of Botany. The university had a 50-year history of excellence in the field and I took plant morphology courses from Barbara Palser and Paul Voth. I became interested in histochemistry and, through a bizarre set of circumstances, ended up doing my doctoral research at the Carlsberg Laboratory in Copenhagen, Denmark with Professor Heinz Holter, although the degree is from the University of Chicago.

At that time, I was interested in cell development in root tips and was using the histochemical procedures of Linderstrom-Lang and Holter. Realizing I did not have enough biochemistry to do the kind of research I was interested in pursuing, I went to the California Institute of Technology on a post doctoral fellowship. There I worked first with Art Galston, and later with James Bonner. This was a very exciting time in my life and a time whose events influenced me greatly. I went back to Europe to be in Jean Brachet’s laboratory in Brussels, primarily to learn autoradiography, techniques that were just being developed at that time. I returned to the United States to become an assistant professor at the University of Virgina. I was there only a year when I was hired by the University of California, Berkeley.

Berkeley was a fantastic place for a young professor. I was a member of the Department of Botany and had as colleagues men who were the best in their fields and wonderful characters as well. It was the late 1950s and science in the United States was exploding. Research money was available and everything was “full speed ahead”. Molecular biology was just taking off and new exciting techniques were appearing every day.

At Berkeley, I first continued the work I had been doing on root tips and the question of cell differentiation. But slowly I became interested in the phenomenon of cell differentiation in embryos. I selected cotton to study because of the large number of embryos per flower and the fact that the embryos were large. We could isolate embryos of all but the youngest stages and analyze them for substances like proteins and nucleic acids. Using a microrespirator developed at the Carlsberg Laboratory, the Cartesian diver, we measured oxygen uptake of the developing embryos.

At this point the electron microscope entered my life. Today it is difficult for many young investigators to understand the impact of the electron microscope, but it was enormous. There was an overwhelming sense of awe at being able to see so much. Organelles in the cell, which earlier had looked like featureless blobs, were now revealed to have beautiful internal structure. New cell parts now appeared that no one had expected. It was a time of awe and fascination.

Yet, the research was not that easy, particularly with plant cells. Fixation was difficult and never very certain. Sectioning using glass knives was horrendous; the knives never stayed sharp and made vicious stratches. But, for all of that, you could see things, as murky and scratched as they were, that you could never see with the light microscope. Finally, slowly we began to see things on a regular basis and we began to understand the ultrastructure of the cell. Suddenly, breakthroughs were occurring routinely and new techniques were being worked out. One example involved Katherine Esau and Keith Porter. The three of us were on a large committee to study the future of botanical research. Katherine and I had been working with permanganate fixation and we were both pretty proud of the results. Porter was unable to attend the committee meeting, but he wrote a letter stating his opinions. At the bottom of the letter he had written a brief comment to the effect that we should stop using permanganate fixation and switch to glutaraldehyde osmium, as it was far superior. I remember Katherine looking at me and saying, in effect, “In no way am I going to change my fixation. It has taken me years to work out permanganate fixation and that is that!“ I agreed with her enthusiastically, but 6 months later we were both using the “new” fixative and delighted with the results.

Well, in we blundered with our powerful new toy, the electron microscope, to look at the embryo sac and double fertilization. When everything worked well, we could see everything; when it didnot, as was often the case, we saw little or mistook one thing for another. For example, we thought for a while that the cotton embryo sac had a single synergid because we simply could not find a second in our preparations. What in the world we thought had happened to it is lost in the fog of the time, but we were surely wrong. Other things worked out better.

We were among the very first to see sperm in the pollen tube with the electron microscope and show they were real cells (Jensen and Fisher 1968b). True, they were not the world’s greatest cells, but they had plasma membranes, nuclei and a few mitochondria and vesicles. Coiled around them was the wonderful vegetative nucleus, in cotton, at least, lacking a nucleolus. Among the things that continue to amaze me is the persistent belief by textbook illustrators that the sperm and the vegetative nucleus are separated by vast distances in the pollen tube and their ambiguousness as to the nature of the sperm cells. The majority of biology textbooks still have naked nuclei moving down the pollen tube.

Our major effort was not the pollen tube, but the embryo sac. My biggest surprise was the synergids. We found, in cotton, synergids that were huge, wonderful cells, full of cell organelles and surrounded by one of the most unusual cell walls found in plants (Jensen 1965a). At one end of the cell the wall elaborated into the beautiful filiform apparatus, while at the oppositic end of the cell the wall just disappeared. This, of course, means that there is an area available for the transfer of the male gamete. The egg, in contrast to the synergid, is a cell with a large central vacuole and a low population of cell organelles (Jensen 1965b). It, too, is surrounded by a wall that simply disappears at the chalazal end of the cell. We were amazed to find that the two polar nuclei were connected through the endosplasmic reticulum projecting from their nuclear membranes (Jensen 1964). The central cell, which houses these two nuclei, also possessed a very active cytoplasm.

When we looked at what happened as the pollen tube approached the embryo sac, we were surprised to see one of the synergids begin to degenerate (Jensen and Fisher 1968a). It was into this synergid that the pollen tube grew and discharged the gametes. The pollen tube grew through the filiform apparatus into the degenerating cytoplasm and discharged the gameter through a pore in its side, not at the tip. There were many changes in the degenerating synergid, but the most significant was the breakdown of the plasma membrane. This meant that the plasma membrane of the sperm could come into direct contact with the egg plasma membrane. The same thing was true for the central cell. We never found a sperm fused with the plasma membrane of the egg or the central cell, but we saw sperm nuclei in the fertilized egg and the central cell cytoplasm. We also saw the sperm nuclei fusing with the egg and polar nuclei (Jensen 1968; Jensen and Fisher 1968a).

The most exciting thing about these observations was that we were the first to make many of them. We started working with cotton, but that soon changed: Pat Schulz took on *Capsella* (Schulz and Jensen 1968a–c); Dave Cass did barley (Cass and Jensen 1970); Alferdo Cocucci looked at *Epidendrum* (Cocucci and Jensen

1969a–c); most important of all was Don Fisher, who worked with me on cotton (Jensen and Fisher 1968a, b, c, d). In a furious 3-year period, 1966 though 1969, we published a total of 26 research papers. But we were not alone. Hard on our heels was Professor Linskens and an energetic, bright group of Dutch investigators working with *Petunia*. We seemed to be locked in a headlong race with one group ahead and then the outer, but it was an exhilarating experience. Others were also working on double fertilization and it was a truly exciting time. To make it all the more interesting, it was a period of campus unrest at Berkeley, with riots erupting regularly and tear gas swirling around us. This added excitement to the preceedings, but never seriously impeded the research. This of course, is the hindsight of age, but I do not think I am too far off base.

The research continued after this period in my laboratory, but the intensity decreased. We worked with Bud Beasley with cultured, unfertilized cotton ovules (Beasley and Jensen 1985). We found that the cells of the embryo sac respond to growth hormones in ways that mimic the response due to the pollen tube and fertilization. We got involved with a huge chemical company and looked at the effect of a gametocide compound on wheat pollen Mizelle et al. 1989). I also had two wonderful final postdocs, Gamel El-Ghazaly (El-Ghazaly and Jensen 1986), who worked on wheat pollen wall development, and Ru-Ling You (You and Jensen 1985), who worked on the megagamete and fertilization in wheat. I also had in my laboratory a delightful young South African, Valeria Butler, who worked on one of the world's most unusual plants, *Welwitschia*.

Thirty years after we started, there are still plenty of unanswered questions. The basis of double fertilization, how one sperm nucleus always enters an egg and the other the central cell, remains unclear. What actually directs the pollen tube to the embryo sac is another unanswered question. The list could go on and on. The constant quest for better procedures, particularly methods of preparing the tissue for observation, continues. New and better ways to apply molecular biology to fertilization and embryo development are needed. There is always another question and always another answer. My part in that search for answers is over, but my interest in the answers remains as intense as ever.

In summary, I thank Joe Mascarenhas for asking me to contribute my very personal thoughts on the 100th anniversary of the discovery of the process of double fertilization. I would like to thank those in my own laboratory who made the work possible. First is the ever-faithful Mary Ashton, and then the best electron microscopy technicians I have ever worked with, Paula Setler and Bernice Lindner. I also thank a host of graduate students, including Ed Polloch, Pat Healy, Mike Forman, Bong Yu, Patricia Schulz, Seri Wetzel, Marie Mizelle and Ravi Sethi; post docs, such as Don Fisher and Dave Cass; visitors such as Alferdo Cocucci and Ru-Ling You; and many others. Clearly, my interest in this vital process was kindled at the University of Chicago by the legacy of that institution in morphological research kept alive by Barbara Palser. I hope the people whose names I left out will forgive me, as I mean to offend no one. Finally, I wish all the investigators working on fertilization and embryo development the best of luck and urge them to keep on looking for answers. Good hunting!

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