In 1675, Dutch microscopist Anton van Leeuwenhoek had to grind his own lenses. Today, scientists use innovative technology and techniques to inspect the microscopic world.

Below—Different microscopic techniques produce different views of the same structure. The top micrograph was made with a standard light microscope using differential interference contrast (DIC) optics. The image depicts the late stages of zoospore formation in the zoosporangium, the spore-forming structure of the fungus *Allomyces macrogynus*. The bottom image was made using a scanning transmission electron microscope after the specimen was prepared by high-pressure freezing and freeze-substitution methods. Top right—a video-enhanced digital light micrograph shows a zoospore being released from the zoosporangium. Under normal conditions, each zoospore has a single nucleus and a flagellum, a long whiplike structure used for swimming.
It sounds like a straight-forward process, but the machine that carried out the freezing is an hpm 010, a $170,000 example of engineering precision. Only nine American institutions have one of the high pressure freezers. Researchers at Arizona State University are the first scientists in the West to have access to such a device.

The high-pressure freezing device represents one of the most sophisticated ways to prepare organisms for viewing under the electron microscope. As part of the Life Sciences Electron Microscopy Facility, it is the latest addition to a wealth of high-tech tools a su biologists are using to unlock the secrets within the microscopic underworld.

The tools range from computer-aided observation to innovative methods of preparation, and they encompass both light and electron microscopes. All are part of a current brand of technologies that, within the last decade or so, have made it increasingly possible for life scientists to study their minuscule subjects in almost undisturbed and increasingly accurate detail.

Using rapid freezing methods for electron microscope studies allows researchers to see and study their specimens in a more true-to-life form.

"Theoretically, we could thaw the cells after we freeze them and they would still be alive," explains Roberson, director of the electron microscopy facility and associate professor of botany.

Roberson’s gold-rimmed glasses rest on his forehead above his eyebrows, where he is apt to lift them anytime he is about to peer into a microscope. He’s been doing this kind of work for more than a decade, mostly studying fungi. He’s convinced that the high pressure freezer is one of the best methods available for preserving large samples of living tissue.

**Tinkering With The Tiny** Preparing a living thing for viewing under an electron microscope is not a simple task. Electron microscopes produce their images by transmitting beams of electrons through a sample and, much like casting a shadow, an image of the specimen is generated on a special screen.

For the procedure to work, the specimen must be placed in a vacuum; otherwise, air and other molecules would deflect the electron beam into a useless scatter. Under normal circumstances, such an evacuated chamber would blow a cell apart. To compensate, scientists must stabilize their cells and tissue specimens.

Traditionally, electron microscopists prepared specimens by submerging them in chemicals which formed stable links between the molecules of a cell’s internal structures, or organelles. The process, called fixation, preserves the subcellular architecture so the tissue can be prepared for viewing. Samples are then usually embedded in an epoxy resin, sliced ultra-thin with a diamond knife, and placed on a metal grid.

Despite its pivotal role in microscopic studies, fixation also comes with a trade-off. The same chemicals that immobilize cellular components sometimes interact with them as well, causing abnormalities in their shape and position.

"With chemical fixation, depending on how thick the tissue is, it takes anywhere from 30 seconds to five minutes for a fixative to penetrate the tissue," explains Mike Harding, a graduate student in Roberson’s lab. “Meanwhile, the cell reacts to the slow rate of fixation in an adverse way, and probably is doing all kinds of crazy things. So what you see may not be representative of what the specimen looked like prior to being fixed,” Harding adds as he prepares to study root fungi with the electron microscope and the hpm 010.

The high-pressure freezer solves some of these problems and makes it much more likely that the way a cell looks after it is prepared is consistent with its prefixation state.

Indeed, since a su acquired the hpm 010 in August 1994, Roberson and master’s student David Lowry conducted experiments that indicated some molecules involved in the process of maintaining cytoplasmic order were much more numerous in cells fixed with the rapid high-pressure freezer than in cells fixed using conventional chemical methods. Roberson attributes the difference to chemical degradation in the traditionally-prepared samples.

The high-pressure freezer blasts cells with a stream of liquid nitrogen which, at 197 degrees below zero Celsius, stops life processes within milliseconds. In the life of a cell, that translates to an almost instantaneous arrest of biological activity.

The result: a cell’s metabolism is essentially suspended, but the cell has not yet died. By using a process called “freeze substitution,” scientists can then use chemicals to lock the frozen molecules in place. But unlike with chemical fixation, they are not relying on the chemicals to stop the biological activity in the first place.

If this description conjures up futuristic images of preserving whole corpses so than an individual might be revived to meet his or her grand-relatives at some far-distant date, nip those dreams right now. Roberson says the high-pressure freezer–and cryogenics in general–work only for very small pieces of tissue.

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Exocytosis happens like the reverse of sticking one’s finger into a balloon and pinching off the mini bubble that forms. Small vesicles, or circles of lipid, encompass cellular substances packaged inside the cell. The substances, usually proteins, are released when their vesicles fuse with the cellular membrane.

Some scientists saw a loop or “bleb” on the outside of the cell’s membrane during this process; while others observed that proteins embedded in the cell’s surface membrane disappeared just prior to the exocytosis of underlying vesicles. The “blesbs” were thought to be an intermediate step in the exocytosis process, but were later shown to appear only if the cryoprotectant glycerin was used.

Chandler and his colleagues showed that the second phenomenon, called protein clearing, appeared in chemically fixed cells, but not in cells that were frozen. Both phenomena were observed for many times, and were so vivid, that Chandler’s peers were skeptical of his conclusions at first. Today, he thinks that their results, not his, were what scientists call “artifact” – a misleading result attributed to experimental manipulation rather than a genuine phenomenon.

Importantly, Chandler notes that whatever caused the artifacts in the first place must have some biochemical cause, but he’s not pursuing the meaning of that process at the moment. “We think we’ve dispelled 80 percent of the doubt that his results were artifact.” But protein clearing was thought to be an important part of the exocytosis process for about 10 years,” Chandler says.

When he began his career, Chandler studied the cortical reaction, a classic example of exocytosis which occurs in recently-fertilized egg cells. A split second after the sperm penetrates the egg, vesicles lying below the egg’s surface membrane fuse and release proteins into the extracellular space.

The reaction was long known to cause the once-receptive egg to become impenetrable to sperm (preventing more than one sperm from inserting its genetic material into the egg—a situation fatal to the new embryo in most cases).

Part of the way the reaction accomplished this was by causing a thin membrane that encircled the egg surface to lift away from the cell. The membrane, called the vitelline envelope, appeared in micrographs of thin sections as a wispy, fairly uninteresting layer bounding the outside of the cell. No one paid much attention to it. Until one day, when Chandler viewed the vitelline envelope using a new technique.

Chandler was then a post-doctoral student studying sea urchins. At the time, his mentor was developing a process called deep etching. Prior to that, samples were commonly freeze-fractured, meaning frozen and then cracked open. The fracture usually happened along the line between the two layers of a cell’s surface membrane. The outer half of the membrane— and the vitelline envelope that surrounded it—was usually washed away to reveal the detail of the membrane’s interior.

In most cases, the “freeze” part of freeze-fracture was accomplished by dipping the samples into liquid freon, liquid propane, or some other cryogen. But “plunge freezing,” as it is called, is fairly slow, so glycerin is usually added to prevent ice crystals from forming.

The deep etching technique hinges on a process called “sublimation,” which cannot occur in the presence of glycerin. So, instead of plunging samples into cryogen, Chandler slammed them against a super-cooled mirror or block of copper, which sucked the heat out in a matter of milliseconds.

Then water was sublimed, or evaporated, by going directly from the solid to the gaseous phase. Gradual sublimation exposes the three-dimensional surface of a sample from the outside inward, like an ice block melting away to reveal a solid sculpture within.

The sample is then sprayed with metal from all sides, and the original specimen is washed away to reveal a three-dimensional, platinum “replica” of the specimen that can be viewed under the microscope.

Because the whole specimen was intact, Chandler was able to view the vitelline envelope in detail. What he saw was a vast network of interwoven proteins above the cellular membrane that seemed intricately complex. More importantly, it drastically changed its appearance and structure in response to the release of proteins during the cortical reaction.

The network, the vitelline envelope, was an example of extracellular matrix—proteins that exist outside of cells and link them together or perform other important functions.

The metamorphosis Chandler observed in the extracellular matrix of the sea urchin egg changed the direction of his career and others’. People began to study exactly how the vitelline envelope changed to perform the two completely different functions of enticing sperm into and keeping sperm out of the egg.

“It was a surprise finding in the sense that I wasn’t really interested in the extracellular matrix,” Chandler recalls. “People talked about it. But I just routinely decided to deep etch my exocytosis stuff. I can’t remember why exactly—probably just to get a different view of the exocytosis process.

“Suddenly we saw the pictures, and I said, ‘Holy cow, what is that?’ Then it dawned on us and we said, ‘Of course. That’s the stuff you can hardly see in thin sections that people think is
important for sperm reception. We immedi-
ately knew something fantastic was there.”

Chandler has since become expert at the
slam-freeze/deep-etch process, which few
people in the world bother to tackle.
“It’s enough of a pain that not many people
do it routinely,” he explains, “even though it’s
widely recognized as one of the best ways to
get the best possible preservation.”

Chandler also has pioneered the under-
standing of egg extracellular matrices and
characterized the precise biochemical changes
of the vitelline envelope in both sea urchin and
frog eggs.

Using microscopy and biochemical studies,
he has shown that the vitelline envelope
changes from a loose network of proteins into
an armor-like coating that protects the egg, both
from further fertilization and probably from
infectious agents such as viruses and bacteria.

Most of Chandler’s work has been done
on pieces of egg. Now he wants to use the
high-pressure freezer to study a different kind
of extracellular matrix. This one resides on
the inside of early embryos and guides the
continuously dividing sheets of primordial
nerve, muscle, and gut cells into their final
positions in the developing fetus.

For this work, he wants to view whole sea
urchin eggs which, at a diameter of 0.5
millimeters, are too big to slam freeze without
being littered with ice crystals.

“The high pressure freezer is the only
technique (other than chemical fixation) that
can freeze a sample that big for electron
microscopy,” Chandler explains.

Indeed, the ability to effectively prepare
thick specimens without chemical artifact is
one of the greatest advantages of the high-

Doug Chandler’s fingertip displays a tiny gold grid used to hold specimens that will
undergo freeze fracture. Magnified more than 50,000 times with an electron microscope,
freeze fracture techniques result in richly detailed, artistic images. Seen here is the outside
portion of a sea urchin egg known as the cortex. The fracture plane goes directly through
the egg’s plasma membrane. The large secretory granule at bottom right is actually less
than 1 micron in diameter, about a thousandth of a millimeter across.
pressure freezer. It’s partly what drew graduate student Mike Harding to a su from Canada.

Harding wants to study a fungal-plant association called va m, or vesicular arbuscular mycorrhizal fungus. Like all mycorrhizal fungi, va m grow inside or near plant roots. Although micorrizal relationships are not fully understood, scientists generally believe that the pairing is a mutually beneficial one, with the fungus providing hard-to-get-at nutrients like phosphorus to the plant in exchange for the photosynthetic byproduct carbon, which the fungus uses as food.

More than 90 percent of the Earth’s plants form associations with mycorrhizal fungi, including virtually all forest species and almost every agriculturally important plant. Still, a great deal remains to be discovered about the dynamics of the fungus-plant relationship.

Some unanswered questions include: Do both parties always benefit from the association? Who benefits the most? Does the relationship change from mutually beneficial to exploitative during periods of stress and food shortage? How might such a shift affect crop production or forest growth during, for example, times of drought?

To answer some of these questions, Harding intends to study va m arbuscules, convoluted structures of membrane that serve as nutrient exchange organs. He will start by growing va m in the roots of bell peppers and characterizing the appearance of the root-arbuscule interface. Then he will subject the plant-fungus system to various stressors, including heat, to see how the relationship changes.

The magnitude of his task is monumental. To study the arbuscules, he must first find them. Although va m can grow in about 80 percent of a plant’s root, arbuscules only form in a portion of the fungal mass.

Moreover, each arbuscule is a complex three-dimensional structure that is only present for between eight and 10 days, and only one part of the arbuscule is the focal point for nutrient transfer. Harding says that if he had to search through root tissue in search of arbuscules using specimens one or two cell layers thick, it would be impossible.

“I wouldn’t even try it,” he says.

But because the high-pressure freezer allows him to freeze samples up to half a millimeter thick, Harding says he can realistically use cryotechniques to visualize the root system in search of the appropriate structures. He can then use thin sections to reconstruct the structures in three dimensions.

The secret to the h pm 010’s success at freezing that all-elusive thick sample? Pressure. And timing.

The liquid nitrogen is fired at the sample using a blast of pressure equivalent to 300,000 pounds per square inch—the same force as the blast from the barrel of a 30.06 rifle.

At that pressure, a living sample would be blown apart. But the extra-cold nitrogen reaches the sample a fraction of a second before the pressure, and the specimen is frozen solid before it can be harmed.

The high-pressure freezer is also effective because it outsmarts water. At that pressure, water is frozen in a vitreous, or glass-like state. Thus, the absence of ice crystals.

The process is not fool-proof. In fact, it takes considerable practice to carry out any of these highly-sensitive fixation processes.

Quing Fang He, an a su doctoral candidate in botany, knows this all too well. His object of interest is a bacterium called Synechocystis.

Under Roberson’s guidance, He has been trying to fix samples of Synechocystis so that he can study how the cyanobacterium mobilizes proteins that regulate the expression of the photosynthetic pigment chlorophyll in response to changes in light. But he keeps getting ice crystals.

“It’s not simplicity that counts. It’s the results,” says He’s advisor, Willem Vermaas, a su professor of botany.

One of the most powerful advantages of the high-pressure freezer is that it maintains the chemical and structural integrity of most biological molecules in a specimen. For He, that carries the promise of a good view of complex photosynthetic membranes that are difficult to see in chemically fixed tissue.

It also means that scientists can combine traditional, structural studies of cellular space with state-of-the-art functional techniques. That kind of advance extends the utility of the microscope, an instrument whose basic physical and optical principles have remained essentially unchanged since the middle of this century.

“Methodology to a great extent drives any science,” says Allan Bieber, director of ASU’s molecular and cellular biology program. “Whenever you get a significant advancement in methodology, you usually see a corresponding jump in knowledge.”

The jump can be astounding when investigators combine multiple methods, as Robby Roberson does. Using light microscopy, conventional and freeze-prepared electron microscopy, computer-aided viewers that enhance contrast, and video microscopy, Roberson is piecing together the process of apical growth in the fungus Allomyces.

Apical growth occurs at the ends of fungal hyphae, but it is analogous to other growth processes as well.

“Apical growth is fairly unique in eukaryotes (non-bacterial organisms). But nerve cells and root hairs do it as well,” says Roberson.

In 1992, Roberson and graduate student Margie Vargas happened upon an unexpected sight that triggered his interest in Allomyces.

“It was one of those late nights in the lab, and I saw something no one had ever seen before. Textbooks are going to be rewritten now because of our discovery of that little thing right there,” says Roberson, pointing to a starburst-like structure at the end of a fungal filament.

The structure, called a Spitzenkörper, or apical body, had been described in various fungi. But never before had it been seen in Allomyces, which was always characterized as belonging to a family of fungi that lacked the structure.
The discovery led Roberson to look for a certain protein, called tubulin, in *Allomyces*. Using light microscopy, and cells labelled with fluorescent molecules, Roberson and Vargas have shown that what they think is tubulin appears around the border and near the tip of growing *Allomyces* filaments. When grown in drugs that disrupt tubulin, the fluorescent organization disbands and the cells stop growing, suggesting that this protein might play a role in apical growth.

Roberson and graduate student David Lowry then looked at the cells under the electron microscope to see in detail what another important protein called actin was doing. To distinguish actin from other molecules in the cell, they incubated the cells with antibodies joined to gold particles and that were specific for actin.

The resulting images revealed gold particles clustered in the vicinity of structures called coated vesicles— which are in fact analogous to the vesicles of the cortical reaction. In this case, Roberson assumes that the vesicles interact with the plasma membrane and help the cells grow.

Using the cryo-fixation techniques, Roberson’s group hopes to follow the fungus’ lead and more precisely characterize actin’s role. Eventually, Roberson says, he hopes to come full circle and use the light microscope to obtain pictures of actin’s movement in animated, growing, pure living cells.

Sometimes our simplest instruments give us the most integrated picture.