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PERSPECTIVES

MICROBIOLOGY PIONEERS — ESSAY

Manfred Girbardt and Charles Bracker: outstanding pioneers in fungal microscopy

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Abstract | Midway through the twentieth century, the availability of new and improved optical and electronic microscopes facilitated rapid advances in the elucidation of the fine structure of fungal cells. In this Essay, I pay tribute to Manfred Girbardt (1919–1991) and Charles Bracker (1938–2012) — two individuals who, despite being separated by geography and the restrictions of the Cold War, both made equally fundamental discoveries in fungal cell ultrastructure and set high standards for specimen manipulation and image processing.

Manfred Girbardt and Charles Bracker (FIG. 1) were indeed true pioneers of fungal cell ultrastructure. As I began to reflect on the impact of their work, a touch of nostalgia took me back to the laboratory of the late Marko Zalokar in the Scripps Institution of Oceanography, La Jolla, California, USA, in the late 1960s. Zalokar — a consummate cell physiologist and experimentalist — was not a mycologist per se. However, similarly to many other biologists, he found in fungi considerable opportunities for novel experimentation. His work included some memorable experiments in which living hyphae were turned into centrifuge tubes, which led to the convincing demonstration that the synthesis of RNA in eukaryotic cells takes place in the nucleus¹. I travelled from my academic home at the University of California, Riverside, USA, to visit Zalokar, motivated by a desire to compare notes as we were both searching for a biochemical understanding of fungal growth, especially the mystery behind the extraordinary growth power shown by fungal hyphae.

By 1892, thanks to the analytical work of Max Otto Reinhardt², it was clearly shown that hyphae grew at the tip (this is thought to be the first authenticated example of polarized growth); however, not much more was learnt about hyphal growth until the middle

of the twentieth century, when the availability of new instruments and methodologies sparked a growing interest in deciphering the basis of polarized growth. By the time of my visit, Zalokar³ had already published several papers on fungal biochemistry and cytochemistry, which included some of the first high-quality transmission microscopy of fungi. So, there we were, examining in bewilderment the large montage of transmission electron micrographs (TEMs) that were pasted on the otherwise bare walls of his office. The montage covered a good stretch of a hypha of *Neurospora crassa* from the tip backwards, at high magnification. There were zones within the hypha in which nuclei or mitochondria were concentrated but there seemed to be nothing special about the rather empty tip; this was at odds with our autoradiographic studies on chitin and chitosan deposition, which had shown that the tip was the site of most cell wall growth⁴. Nothing in the TEMs of the hyphal tip gave us a clue as to what could be responsible for, or even associated with, the polarized growth of the hypha. Little did we know that a convincing answer was being assembled by two independent sources, one at the Institut für Mikrobiologie und experimentelle Therapie in Jena, Germany, and the other at Purdue University, West Lafayette, Indiana, USA.

Vesicles: cellular secrets of morphogenesis

In 1969, two extensive articles — one from Girbardt⁵ and the other from Bracker and his graduate student Stanley Grove⁶ — revealed in great detail one of the most basic secrets of fungal growth (FIG. 2). With the best transmission electron microscopy available at the time, they showed how it was possible for fungi to concentrate their growing power to a small region: the hyphal apex. Their TEMs of thin-sectioned hyphae from fungi that were representative of the various classes in the fungal kingdom made it abundantly clear that the growing tips of hyphae contained a highly organized accumulation of secretory vesicles. These aggregates of vesicles constituted the mysterious organelle called the Spitzenkörper, which is a spheroidal body that is present at the hyphal tips of septate fungi and was first observed by Brunswik⁷ in 1924 (discussed below).

The abundance of vesicles in the growth region led Girbardt and Bracker to suggest that these vesicles were the main components of the machinery responsible for the polarized growth of hyphae^{5,6}. A vesicle provides enzymes with mobility inside the cell, so vesicles could, in principle, be the mechanism by which the fungus directs, concentrates or focuses its cell wall-making machinery in a defined area, conferring a spatial dimension to cell wall synthesis. The discovery of vesicles that are capable of moving inside the cell was undoubtedly one of the most important breakthroughs in fungal biology and was a strong starting point for hypotheses on the cellular basis of fungal morphogenesis⁸. So why had Zalokar and others failed to see these fundamental structures in hyphal tips? The answer is perhaps a little ironic: they were too meticulous in manipulating their cells before fixation. As Bracker often stated, apical vesicles are a delicate, ephemeral population that can vanish quickly during the harvesting of a fungal culture.

Although the articles from Girbardt and Bracker^{5,6} have received the greatest recognition for elucidating the cellular basis of apical growth, as often happens, there were others^{9,10} who had made similar findings earlier, albeit on a more limited scale. What made the work of Girbardt and Bracker

Figure 1 | **Manfred Girbardt and Charles E. Bracker.** Portraits of Manfred Girbardt (left) and Charles E. Bracker (right) at the height of their scientific careers.

so compelling was the high quality and the extent of the microscopy. Such refined standards were both an inspiration and a challenge to a whole generation of electron microscopists worldwide, whose work on specimens that are usually difficult to fix made fungal ultrastructure a rich subject for the following two decades.

Two different careers converge

The scientific careers of Girbardt and Bracker converged in the late 1960s as they both embarked on a mission to elucidate

the fine structure of fungal hyphae. Although both scientists reached the same goal, they did it under remarkably different circumstances. For the 50 year old Girbardt, it was the culmination of two decades of progressive research that began in the early 1950s. For the much younger Bracker, the elucidation of the ultrastructure of the fungal hypha and the nature of the Spitzenkörper was an early triumph that was achieved just a few years after he was appointed Assistant Professor at Purdue University.

The laboratory environments of Girbardt and Bracker were entirely different. Girbardt was the head of the cytological unit that occupied most, if not all, of one floor at the Institut für Mikrobiologie und experimentelle Therapie, Jena, in what was then East Germany. His best work took place in the darkest period of the Cold War (BOX 1) when he was mostly isolated from his international community of colleagues. He relied on an army of technicians doing the laboratory minutiae, from growing and harvesting fungi to processing the electron micrograph plates. By contrast, Bracker started his career in the open climate of growth and prosperity that prevailed in research universities in the United States in the 1960s and 1970s. For the hyphal ultrastructure project, Bracker relied on a dedicated doctoral student and a single technician. US government funding for research was readily available and international travel was by then almost routine. Many mycologists, particularly in the United States and the United Kingdom, were able to benefit from rich exchanges with colleagues at international congresses and conferences — a benefit that was sadly denied to Girbardt.

Girbardt initially decided to explore the power of phase contrast microscopy to observe the inner structure of living cells without the use of staining¹¹. He was most interested in following nuclear dynamics in living basidiomycete cells and concentrated almost all of his efforts on a single organism, *Polystictus versicolor* (later renamed *Trametes versicolor*), mostly focusing on nuclear motility and division¹². His observations of *P. versicolor* clarified the behaviour of nuclei as they divide and migrate from one hyphal compartment to the next through a clamp connection, which is a tightly curved hyphal branch that links adjacent compartments. Later, in a series of papers, he endeavoured to correlate optical images with electron microscopic images and these studies shed light on the structure of the kinetochore and the role of microtubules in nuclear division¹³. His finding that a filamentous septal band¹⁴ was a prelude to septum formation laid the groundwork for future discoveries of the molecular structure of the contractile actomyosin ring¹⁵.

Bracker made considerable contributions to our understanding of the fine structure and the development of various fungi, particularly plant pathogens¹⁶. His first triumph, achieved in collaboration with James Morre^{17,18}, was to reveal the fundamental features of membrane differentiation in the hyphae of the oomycete *Pythium ultimum*;

a *Polystictus versicolor* (KMnO₄)

b *Aspergillus niger* (OsO₄)

Figure 2 | **Classical transmission electron micrographs of the hyphal tips of fungi.** **a** | Ultrathin sections of *Polystictus versicolor*. **b** | Ultrathin sections of *Aspergillus niger*. The main fixatives used are indicated in parentheses. Both scale bars represent 1 μm. Figure part **a** is reproduced from Springer and Protoplasma, 67, 1969, 414–441, Die Ultrastruktur der Apikalregion von Pilzhypphen, Manfred Girbardt, Figure 7, Copyright ©1969, Springer-Verlag, with kind permission from Springer Science and Business Media. Figure part **b** is reproduced from J. Bacteriol., 1970, 104, 989–1009, reproduced with permission from the American Society for Microbiology.

this study yielded some spectacular images of internal membrane organization and the differentiation of the endomembrane system and paralleled similar findings obtained by animal cell biologists¹⁹. His work on *Melampsora lini*²⁰ and *Erysiphe graminis*²¹ was also notable, showing with extraordinary clarity the details of the infection process in flax and barley, respectively. Bracker also devoted a good portion of his time to studying the ultrastructural aspects of the life cycle of his favourite fungus *Gilbertella persicaria*^{22,23}.

The Spitzenkörper and the chitosome

Girbardt and Bracker deserve much credit for their role in establishing the existence of two key fungal cell structures and their roles in the growth of fungal hyphae: Girbardt²⁴ for proving that the Spitzenkörper existed and had a leading role in hyphal growth, and Bracker²⁵ for the refined electron microscopy that was essential to establishing the nature of chitosomes, which are the microvesicles responsible for carrying chitin synthase in fungal cells. Evidence that these two structures are intimately related and are involved in the biogenesis of the cell wall was obtained decades later by Meritxell Riquelme and her research team^{26,27} (see below).

The Spitzenkörper. In 1924, Brunswik⁷ noticed that there was a spot inside the tips of haematoxylin-stained hyphae of basidiomycetes that he aptly named the Spitzenkörper (or apical body). He suggested that the spot may have something to do with the apical growth of the hyphae, but he could not prove it — three decades later, Girbardt did²⁴. The phase contrast microscope gave Girbardt the power to see structures inside unstained living hyphae. At the very tip of growing *P. versicolor* hyphae, Girbardt found a dynamic phase-dark spot, the behaviour of which indicated direct involvement in hyphal elongation. In growing hyphae, the Spitzenkörper remained closely associated with the elongating tip but it disappeared when elongation of the tube ceased. Amazing examples of this correlation were recorded first with a still camera and later with the help of 16 mm cinematography. Girbardt's movie showing the development of a clamp connection is an outstanding example of using phase contrast microscopy to its maximum²⁴. The film recorded the eccentric movement of the Spitzenkörper inside the curving short branch, which suggested that the black spot was guiding the tip to curve in a tight circle to form the clamp connection. These observations on the major

Box 1 | Girbardt and the Cold War

This personal anecdote is intended to give a taste of the restrictive atmosphere in which scientists in Eastern Europe survived during the Cold War.

In 1970, while I was on sabbatical in Stockholm, Sweden, Girbardt invited me to visit his laboratory. This was not a simple task for him as it involved obtaining official permits months in advance to allow a visitor from the West to enter and to travel through East Germany. Unaware of the restrictive policies, with my wife, daughter and a new Mercedes sedan, I sailed from the Swedish port of Gothenburg to Amsterdam, the Netherlands, and headed for Berlin. Today, this is a simple task, but back then it required special licence plates to drive through East Germany. As we travelled along the autobahn to Berlin, a remarkable scene unfolded. In East Germany, no driver dared to exceed the 100 km per hour limit. With comic irony, we saw East Germans in their tiny Trabant speeding past irritated West German drivers accustomed to cruising the autobahns at 150 km per hour in their powerful cars.

Divided by the infamous wall, and with remnants of buildings bombed decades ago, Berlin was a powerful experience; an invitation for reflection on the fragility of the civilized world and the stupidity of mankind. Crossing into East Germany through the notorious Checkpoint Charlie, we saw a monumental change. The few vehicles and the sombre-looking people walking along the quiet streets of East Berlin provided a shocking contrast to the boisterous heavy traffic and festive attitude in West Berlin. We set out to explore this socialist country, the centre of world attention at the time and left Berlin on what was to be a forbidden journey — this was not my fault as I did not receive a letter from Girbardt telling me to proceed directly to a specific hotel in East Berlin to collect my permit and papers with precise instructions on the route to take to Jena. As I later learned, we did not have permission to wander off the authorized autobahn route. As we casually drove through small and large towns between Berlin and Jena, we discovered no state secrets, military bases or rioting citizens, only the strange experience of people everywhere turning their heads to get a long astonished look at the forbidden dream Mercedes that we were driving. Imagine our embarrassment when we finally reached Jena and related our travel adventures to Girbardt, who must surely have feared repercussions for the infractions committed by his guests.

The Cold War was a cultural experience for me as a transient visitor but was a nightmare for those who lived on the eastern side of the Iron Curtain and who had to endure many limitations imposed by their governments. One that was particularly devastating for scientists was the continuous denial of permits to travel abroad. This restriction reached a perverse extreme when scientists who did not cooperate with the government were given advance permits to attend scientific meetings in the West, only for them to be rescinded days or hours before the intended departure.

involvement of the Spitzenkörper in apical growth were undoubtedly a strong motivation to elucidate the nature of the dark spot by tackling a new challenge — namely, to master the tricky field of transmission electron microscopy of thin-sectioned fungal cells. A decade later, the work on the fine structure of hyphae was completed.

Girbardt and Bracker characterized the Spitzenkörper as a spheroidal assembly of vesicles, some small (microvesicles) and others much larger (macrovesicles; also known as 'apical vesicles'). There were other components that were less prevalent, including some ribosomes and amorphous material. What remained unclear, and in fact was controversial, from their articles was whether or not there was a correspondence between the Spitzenkörper images observed by phase contrast microscopy and the range of apical vesicles observed by transmission electron microscopy. Bracker and Grove's images clearly showed a central region populated by microvesicles but Girbardt was not so sure and, in fact, his three-dimensional (3D) reconstruction does not show a central core,

something that he later admitted was incorrect (M. Girbardt, personal communication). The current consensus is to consider both the cloud of larger vesicles and the core as components of the Spitzenkörper. This dual stratification of the Spitzenkörper was confirmed by cryofixation or freeze-substitution²⁸, which preserved the structure much better than chemical fixation, and was later expanded into three dimensions by electron tomography²⁹. Furthermore, we now know that the stratification is not only morphological but also biochemical (see below).

The chitosome. The finding that chitin microfibrils could be made *in vitro* by a cell-free extract from a fungus³⁰ opened up an exciting line of investigation — to determine the nature and the location of the enzymatic complex responsible for making these cell wall microfibrils. Using shadow-casting electron microscopy, we found 'granules' (35–100 nm in diameter), some of which were attached to the ends of the microfibrils, and reported them as the source of chitin

Figure 3 | Girbardt's classic three-dimensional reconstruction of the tip of a hypha. Model of the internal organization of the hyphal apex of *Polystictus versicolor*, showing populations of macrovesicles (ApV) and microvesicles (MV) as part of the Spitzenkörper (SpK). ASW, outer mucilaginous wall layer; CM, plasma membrane; CMI, plasma membrane invagination; Cr, mitochondrial crista; ER, endoplasmic reticulum; GC, Golgi cisterna; ISW, inner fibrillar wall layer; MiK, mitochondrial 'krümmung'; Mi, mitochondrion. Figure is reproduced from Springer and *Protoplasma*, **67**, 1969, 414–441, Die Ultrastruktur der Apikalregion von Pilzhypen, Manfred Girbardt, Figure 7, Copyright © 1969, Springer-Verlag, with kind permission from Springer Science and Business Media.

synthetase³¹. Bracker privately questioned our published assertion that the granules were not membranous structures. His scepticism was grounded in his findings on the prevalence of membranous organelles inside fungal cells. He offered to come to Riverside, California, and spend a sabbatical leave in my laboratory to settle the question. Jose Ruiz-Herrera also agreed to join the quest. Better procedures, which mainly relied on density gradient centrifugation to fractionate cell components, were thus designed to isolate the granules. Luckily, the granules could be neatly separated from the rest of the subcellular structures owing to their small size and their low specific gravity. However, it was Bracker's skill and patience in examining the individual fractions from the gradients that revealed that the granules were indeed membranous structures akin to the microvesicles seen in transmission electron microscopy of cells²⁵. Most of Bracker's electron microscopy work involved negative staining and some thin sectioning that showed the unique membrane structure of the granules, which were named chitosomes. Some of the most impressive images were obtained by incubating the electron microscopy grids with chitin substrate

(UDP-*N*-acetylglucosamine) and observing the field of chitosomes covered with microfibrils. Such incubations yielded convincing images of the connection between chitosomes and microfibrils²⁵. The extraordinary quality of the electron microscopy images gave much confidence in the validity of a whole series of subsequent experiments that showed the existence of chitosomes in various fungi.

The thoroughness of the evidence on which the chitosome discovery was based, particularly the unique features revealed by Bracker's electron microscopy, was overlooked by competitors in the field of chitin biosynthesis, who dismissed chitosomes as artefacts³². Recent findings from the Riquelme laboratory^{26,27} have substantiated the original claims that established the chitosome as a unique exocytic vesicle that is responsible for delivering the enzyme to make the microfibrillar skeleton of the cell wall. Confocal microscopy of growing hyphae of *N. crassa* in which chitin synthases had been tagged with fluorescent proteins revealed that the label colocalized with the microvesicles (chitosomes) that were concentrated in the core of the Spitzenkörper. Similar tagging of proteins that are involved in

β -glucan synthesis showed that this enzyme was associated with the macrovesicles in the outer zone of the Spitzenkörper. Thus, the Spitzenkörper was shown to hold, in a stratified manner, the two major types of enzymes needed for cell wall biogenesis: chitin synthases in the microvesicles in its interior and β -glucan synthase in the macrovesicles in its exterior. This duality of form and function poses several intriguing questions that were addressed in a recent review³³.

Skills and innovation

Girbardt and Bracker were not only accomplished microscopists but also passionate technicians who were dedicated to extracting maximum results from their tools and specimens. The methodologies described in their publications are examples of the effective use of new technologies.

Pioneering efforts in microscopy. Girbardt was one of the first biologists to take advantage of phase contrast microscopy for the observation of living cells. By carefully matching the refractive index of the surrounding medium to that of the cell surface, he revealed elaborate internal structures in fungal cells that showed almost no internal structure by bright-field microscopy¹¹. Girbardt tested cinematography to make a convincing record of his observations²⁴. This was then a cumbersome task but it enabled him to have permanent records of living cell behaviour regardless of the inconvenience of waiting for the film to be developed. Girbardt's efforts in operating a 16 mm movie camera attached to the microscope are remarkable, especially when one compares it with the ease of recording motion with current digital equipment. Girbardt also ventured early into the challenging field of electron microscopy. His 1958 article on the fine structure of *P. versicolor* was one of the first publications with good images of thin sections of fungal cells captured by transmission electron microscopy³⁴. He later devised a practical method to examine the same cell region by phase contrast microscopy followed by thin-section electron microscopy³⁵.

Video microscopy. In the 1990s, Bracker and his student Rosamaria Lopez-Franco pioneered the use of video technology for studying the behaviour of living fungal cells. The phase contrast images captured by the video camera were manipulated electronically to enhance the contrast and were fed to a recording device (in those days a VHS tape recorder). The combination of electronics and microscopy liberated researchers from

the tedium of being stuck to the eyepieces of the microscope. A digital image on a computer screen made it practical to make and record extended observations of living cells. Two important applications resulted from this technology. First, by mapping the movements of the Spitzenkörper with great precision, a crucial test³⁶ was given to the hypothesis that the Spitzenkörper functions as a vesicle supply centre (VSC), as predicted by the hyphoid equation (an equation describing the generation of an apical gradient of growth by the continuous discharge of vesicles from a forward moving source — the VSC)³⁷. Second, precise measurements of hyphal elongation rates every 1–5 seconds led to the discovery that hyphae grow in pulses³⁸.

Laser tweezers. In his final years of active research, Bracker became interested in the application of new tools. Together with Lopez-Franco, he travelled to the Beckman Laser Institute of the University of California, Irvine, USA, to venture into the field of laser microbeam manipulation. Their goal was to use laser tweezers to grab the Spitzenkörper and test ideas on its role in hyphal growth. However, the Spitzenkörper evaded the laser field. Instead, other cell particles were trapped and they could be moved at will and used to ‘push around’ the Spitzenkörper³⁹. By deliberately moving the Spitzenkörper, bizarre hyphal shapes were generated, proving experimentally that this structure directs hyphal growth, as Girbardt had predicted by observing the normal behaviour of the Spitzenkörper.

The quest for perfection

In both Girbardt and Bracker, we find two investigators who were intent on perfecting

their techniques to produce results of the highest possible quality. The Girbardt I met in Jena was the leader of a group of technicians who were specialized in different aspects of the hyphal ultrastructure project, from cultivating, harvesting and fixing various mycelial fungi to the more demanding tasks of sectioning and electron microscopy. His proudest achievement was in a dedicated room labelled ‘Stereologie’, where a spatial model of a hyphal tip was painstakingly put together (discussed below).

Bracker was well known in the international mycological community for the excellence of his micrographs and the meticulousness of his publications and conferences. In an effort to attain maximum fidelity in his images, he went to enormous lengths. For years, colleagues at annual meetings enjoyed telling and listening to anecdotes about the shows Bracker mounted. At the first International Mycological Congress in Exeter, UK, in September 1971, Bracker made the debut presentation of his hyphal ultrastructure work. He would not trust the local projector and, at great effort and expense, brought his own equipment with him; not the 35 mm slide projector that everybody else used in those days, but rather his own 4" x 6" lantern projector, a behemoth of a machine, together with a large collection of heavy glass slides. In addition, he would not trust a local assistant to show the slides, so he also brought his trusted technician Lina Montecillo to keep the images in perfect focus. The quality and the novelty of the micrographs on the screen dazzled the audience but left the unlucky speaker that followed him on the programme obliged to begin his talk by apologizing profusely for the ordinary quality of his own slides.

Regretfully, this incessant search for perfection took a heavy toll on Bracker's physical health. It was also an inhibiting factor that prevented him from publishing some important accomplishments in greater detail — for example, his work on the disassembly and reassembly of chitosomes⁴⁰ or on the manipulation of the Spitzenkörper with laser tweezers³⁹. He argued that these studies were incomplete and feared going public.

Two iconic images. Two figures from Girbardt and Bracker's publications typify their creativity. Girbardt's 3D reconstruction of a hyphal tip⁵ was made from dozens of TEMs copied onto plastic plates that were then stacked together to reconstruct the 3D image of the cell interior (FIG. 3). This model vividly shows the internal complexity of the fungal cell in ways that are not obvious from the examination of 2D images. Apart from the spheroidal aggregate of vesicles that make up the Spitzenkörper, there are other striking features; for example, the mitochondria are not the oval structures one assumes from 2D micrographs but are long intertwined ‘sausages’. Equally impressive is the elaborate endoplasmic reticulum network, with tubules and cisternae filling the cytoplasm everywhere except at the apex. Girbardt's model has been reproduced many times for its strong instructional potential. Bracker's spectacular seamless montage of electron micrographs, showing the cytological organization of a single hypha in median section¹⁸, has remained mostly unused because of its very nature (FIG. 4) — it requires a four-page foldout to show a hypha 128 µm long at a magnification of x5000. The montage shows in exquisite detail the cytological gradient that exists in a hyphal cell from a tip full of

Figure 4 | **Bracker's uninterrupted view of the organelle gradient in a hypha.** A seamless montage of near-median sections of an entire hyphal segment of *Pythium ultimum* ~128 µm in length is shown at the top and

enlargements of selected regions are shown at the bottom. Image is reproduced, with permission, from An ultrastructural basis for hyphal tip growth in *Pythium ultimum*. *Amer. J. Bot.* **57**, 245–266 (1970).

vesicles to a zone of extensive vacuolation at the other end and mitochondrial and nuclear regions in between.

Epilogue

The golden years of ultrastructure research championed by Bracker, Girbardt and many others marked an exciting period of fungal biology that came to a rapid decline around the 1980s, when the avalanche of molecular biology massively drained funding and personnel from the rest of biology research. Confocal microscopy of living fungi genetically modified with fluorescent proteins now dominates fungal biology. However, as spectacular as some of these images are, in my opinion they often lack the resolution that is needed for full interpretation. Herein lies my expectation of a renaissance of electron microscopy using improved methods for molecular detection and precise identification of the intracellular location of metabolites and macromolecules.

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Competing interests statement

The author declares no competing interests.