THE MICROSCOPE
Vol. 70:4, pp 163–176, 2023
https://doi.org/10.59082/QGBV8515

CRITICAL FOCUS

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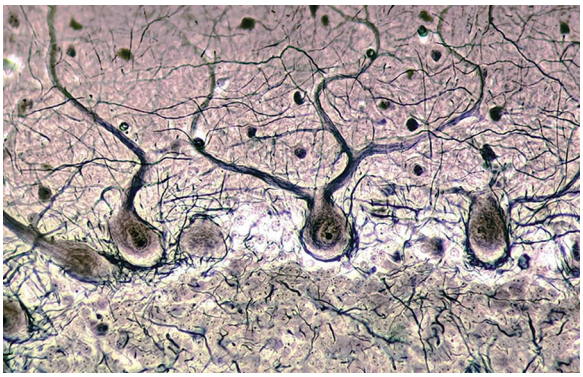
Microscopy's Ingenious Legacy

Beside the high-tech gadgetry in today's laboratory reside the basic stains, reagents, and microscopical techniques that date back to more than a century—and remain essential.

The history of microscopy is often seen as a specialist subject, and one that doesn't influence us working in today's busy modern laboratories. But the history is closer than you think. Look around you. Many of those stains that you take for granted are ancient history. Those computers we use, our mobile phones, the digital equipment, and all that glass and plastic gadgetry are new (indeed, by the time you've purchased and installed them, they are already out-of-date). But not the essential stains and reagents. Many have unexpected origins, and some of them tell extraordinary tales.



Czech microscopist Jan Purkyně investigated the structure of the brain in 1837, revealed in this study of canine cerebellum by Lennart Rikk. This modern section is stained with hematoxylin, known to the Mayans for thousands of years and now a leading microscopical stain, and eosin, named in 1874 after its discoverer's girlfriend.

The first use of a stain to study a microscopical specimen dates from 1714, when the pioneering microscopist Antony van Leeuwenhoek used saffron to dye striated muscle. He was comparing muscle tissue from an old, fat cow with that from a younger, leaner animal, and his limner found it hard to discern the details. Leeuwenhoek described what he did in a letter to the Royal Society: "The fibrillae, cut into the thinnest possible layers, were so transparent that they could hardly be recognized, so I macerated a little saffron … to make the fleshy particles more visible to the artist. I merely moistened them with this wine, whereupon they were bright with a yellow color." Already in 1684 the French anatomist Raymond Vieussens had injected saffron dye to demonstrate blood vessels in the brain, though Leeuwenhoek was the first to use a stain to increase visibility of a microscopic object.

Saffron is a yellow dye obtained from the vivid, orange stamens of *Crocus sativus*, autumn crocus* and, because of its subtle flavor, it is also widely used in specialty cookery. The name is from the Arabic زَعْفَران, za'farān, and saffron was known to the ancients who first cultivated it some 3,500 years ago.

Belize features two baymen on its coat of arms, indentured laborers who collected lumber by felling the logwood tree, *Haematoxylon campechianum.* The purple dye that appeared when the wood was exposed to air was used on textiles, but it tended to fade, and for many years its use in England was unlawful. It eventually emerged as an important microscopical stain.

After saffron came carmine. It proved to be of importance in microscopy in 1770, with John Hill and his studies of wood histology. Hill was borne in the cathedral city of Peterborough and used carmine to improve the appearance of his fine-cut sections. Carmine has a satisfyingly scientific sound to it, yet it originates, not from the experimental laboratory, but from the ancient Aztecs some 2,000 years ago. They obtained the red dye from the crushed bodies of the cochineal insect *Dactylopius coccus*. The name itself derives from ancient Persia, where they had extracted vermilion from the scale-insect *Kermes vermilio* (in Persian, kirm, hence carmine). I have used borax carmine to stain chromosomes, and it provides excellent results for today's microscopist.

The commonest microscopical stain for tissue sections is hematoxylin. It provides wonderfully crisp and detailed images of subcellular structures, most notably nuclei, which it colors deep blue or purple. It also reveals calcified structures and keratin. Hematoxylin is alkaline and positively charged whereas substances like DNA (and, to an extent, the RNA in ribosomes) are acidic and, because of the phosphate framework of these molecules, they bear a negative charge. As a result, the dye binds strongly to the genetic components of the cell and is the most popular of all routine nuclear stains.

It's not new. Once again, it has an ancient history, and its name provides the clue. It is extracted from the tropical leguminous tree *Haematoxylon campechianum*, which grows extensively in Middle and Central America. The genus derives from the Greek signifying blood (αίμα, haemas) and wood (ξύλο, xylos) — lumber, the color of blood. It is another legacy from the ancient Mesoamericans, who had used it for thousands of years. The species became known to the Spanish, who called the tree Palo de Campeche, after the bay in which it was found. Its stems are covered with thorns, and it is often grown as a hedging plant. The wood was being used to as a dye by Mayans on the Yucatan Peninsula when the Spanish conquistadores arrived in 1502, and to European eyes this was a miraculous revelation. The only purple dyestuff in regular use was indigo, produced by fermenting the leaves of *Indigofera tinctoria.* The color of hematoxylin was more vivid. Since the color purple was traditionally associated with royalty, the dye became of great commercial value. We know of pirate ships attacking cargo-carrying vessels for their gold, but the logwood was just as eagerly seized. The great English explorer (and buccaneer) Sir Walter Raleigh would lurk near the Spanish Azores in the mid-Atlantic, only to steal up on the heavily laden Spanish sailing ships and seize their cargo of logwood.

Although hematoxylin was widely tried as a dye for textiles, it faded in sunlight and was not colorfast. Indigo was less likely to lose its color. So, to protect the nation's reputation, the British government under Queen Elizabeth I banned the use of hematoxylin in a decree that read: "That logwood, of late years brought into this realm, is expressly prohibited to be used by dyers, the colors thereof being false and deceitful to the Queen's subjects at home, and discreditable beyond the seas to our merchants and dyers." Between 1581 to 1662 in Britain, it was not permitted to use it as a dye. Only when mordants were discovered was the dye made more permanent, and the restriction could be abandoned.

Although we think of hematoxylin as a vivid purple color, it is colorless when fresh, and acquires its color only as it oxidizes in air. During the 18th century,

*This species *Crocus sativus* must not be confused with another autumn crocus that looks remarkably similar, *Colchicum autumnale*, which is highly poisonous and must never be used in the kitchen. The toxic component is colchicine, used in small amounts to treat gout, and known to microscopists for its ability to halt mitosis in dividing cells, which aids the study of metaphase chromosomes.

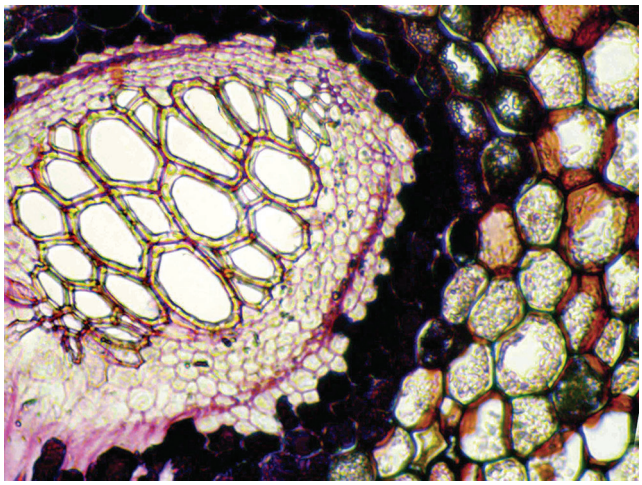
the Spanish claimed a monopoly over the trade in logwood. Many nations (notably the British, French, and Dutch) were eager to exploit this new dye, and English adventurers meanwhile discovered *Haematoxylin* trees growing in British Honduras (now Belize), and warring factions soon emerged over the rights to the timber. The disputes over the lumber gave rise to the Seven Years' War of 1756–1763.

The Treaty of Paris, signed in 1763, gave rights to the British to collect the logwood, though sovereignty in the discovery remained with Spain. The teams of men hired to collect the lumber were known as baymen, and to this day, the national crest of Belize bears depictions of two baymen, harvesting the logwood. As an item of commerce, hematoxylin remained popular, indeed, it was used to dye the fabric from which soldiers' uniforms were made during the American Civil War.

EARLY MICROSCOPY STAINS

Hematoxylin was proposed as a stain in microscopy by Georg Christian Reichel, a member of the German science academy Leopoldina, founded in 1652. He proposed using hematoxylin to stain plant tissues for microscopy in 1785. The pioneering English microscopist John Thomas Quekett recommended the use of the same logwood dye for transparent objects in 1852, and Heinrich Wilhelm von Waldeyer-Hartz pioneered its use for zoological specimens in 1863. Franz Böhmer then published a method for fixing hematoxylin using alum as a mordant, which gave the dye permanence, and by the end of the 19th century, hematoxylin was being widely used in microscopy. Yet it owes its origins to the Mesoamericans before recorded history began.

The Portuguese found another source of dye in several other leguminous tree species growing in Central and South America. The Palo de Tinto tree, *Haematoxylum brasiletto*, was found growing in Mexico and Guatemala. Other trees of South America, along with *Paubrasilia echinata, Biancaea sappan*, and *Caesalpinia violacea*, were also found to yield the same color dye. It is now known in commerce as Natural Red 24 and is a fine microscopical stain, though the deeper color provided by hematoxylin has prevented the alternative becoming widely adopted. The deep red color of the heartwood from these trees inspired the comparison with embers of a glowing fire. In Portuguese, this would translate as *brasa*. They called the dye brazilin, and named the country Brazil. So a stain gave its name to a nation.



The royal fern, *Osmunda regalis*, was the topic I chose for my senior school project at the age of 16. This section of a root was cut by hand using a traditional razor. To show the vascular cambium and inclusions in the cortical cells, I stained it with hematoxylin, a dye first imported in the 1500s. It is not normally used for botanical sections but displays the histology well enough.

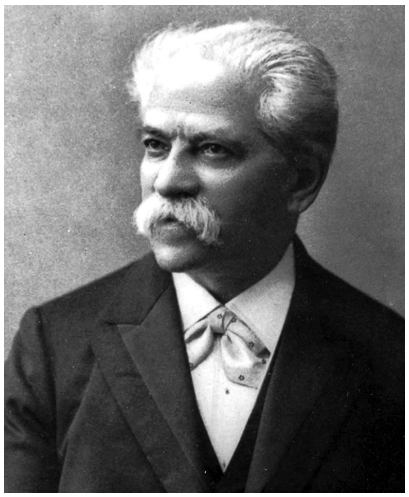
Even before the dawn of the 1800s, microscopy had a range of stains available though they were not designed for microscopists. The aim was to create dyestuffs for textiles, because that was where the money lay. Prussian blue had originally been created in 1704 by Johann Diesbach, who studied dyes in Berlin. He was attempting to devise a red lake pigment which he combined with iron sulphate and potash, but his supply of potash was impure and contained traces of animal fat. Instead of the vermilion for which he was aiming, he was surprised to obtain a bright purple dye which turned deep blue when concentrated. Diesbach knew he was on to a winner. The only deep blue pigment available at the time was ultramarine, and that was fabulously expensive. Ultramarine was obtained by grinding lapis lazuli imported from Afghanistan, which was rich in blue cubic lazurite. This was the color that renaissance artists used to paint blue robes, like those worn by the religious figures they portrayed. Within a few years, Prussian Blue had replaced ultramarine at a fraction of the cost, and it has remained a popular color for artists to this day. It confers the vivid marine blue to the famous woodblock creation by the Japanese artist Katsushika Hokusai, the Great Wave off Kanagawa, surely the most famous of all Japanese art in the West. Gainsborough used it to effect, as did Monet and Constable, and this was the same pigment to which Picasso resorted in his famous "blue period." In today's microscopical laboratory? Why, we now use it to diagnose microscopic deposits of iron

in conditions like hemosiderosis. In just the same way that Diesbach experimented with the aim of creating a valuable new color, other experimenters began working towards the same end.

The dawn of the industrial age following the invention of efficient steam engines by British pioneers, including James Watt and Richard Trevithick in the late 1700s, meant that industrial production was becoming a reality, and the first factories had been born during that era. Large mills producing vast amounts of textiles were springing up across the North of England. The early 19th century brought with it an unprecedented hunger for science and technology, so the quest for dyes became a preoccupation of several inventors.

German chemists emerged as active pioneers. In 1826, a 20-year-old student, Otto Unverdorben from Dresden, extracted a new purple dye by distilling natural indigo. He called it crystallin. Friedlieb Runge, in Hamburg, obtained a deep blue product in 1834 by treating a distillate of coal tar with lime chloride and named it cyanol. Then in 1840, Carl Julius Fritzsche in Berlin treated indigo with potassium hydroxide and obtained an oily blue substance that he called aniline, and two years later in St. Petersburg, Nikolay Nikolaevich Zinin distilled a compound he called benzidam. In fact, the chemistry for each was the same.

It took a brilliant German chemist, August Wilhelm von Hofmann, to prove the point. Hofmann was a pioneer of organic chemistry and came to the notice of Queen Victoria's German husband, Prince Albert. The new impetus for science was something that the Prince felt should be familiar to everyone, and he spent much time establishing institutions to promulgate the new discoveries. In 1845, Albert set up the new Royal College of Chemistry and invited Hofmann to become its first director. One of Hofmann's students was William Henry Perkin, a gifted young student from the East End of London who enrolled at the age of 15. Hofmann taught Perkin about organic synthesis and said he was hoping to find a way to synthesize quinine. This compound was extracted from the bark of the fever tree *Cinchona*, a genus native to South America, and the only known treatment for malaria. It was widely used by indigenous tribes long before Europeans crossed the Atlantic, and the trees were always known as quina-quina to the Quechua people who lived throughout South America. The plants were exported by the British to colonial India and were used to prepare anti-malarial concoctions. Since the taste of quinine was bitter, the British used to blend it with citrus juice and gin to make it more palatable — the reason why a gin and tonic is still a popular beverage in Britain. Though most people don't realize the fact, it's a reminder of Britain's colonial traditions.



Heinrich Caro invented eosin in 1874, naming it after his girlfriend. Methylene blue was his next invention in 1878, another microscopical stain that is widely used throughout the world. Its chemical composition also confers therapeutic benefits, and it has been used to treat malaria, carbon monoxide and cyanide poisoning, septic shock, and even Alzheimer's disease.

Hofmann suggested to the young Perkin that N-allyl toluidine ($C_{10}H_{13}N$), obtained by distillation from coal tar, could be a good starting-point for conversion to quinine ($C_{20}H_{24}N_2O_2$). Easter was approaching, and the enthusiastic young Perkin continued experimenting during the vacation in the attic of his family home in Cable Street, London. He was just 18 when he made an epoch-making discovery. Perkin was trying to synthesize quinine by treating aniline with potassium dichromate and sulfuric acid, but no quinine appeared. Instead, all he had in his test tube was a dark, tarry, sticky substance. He could not wash out the tube with water to start over but noticed that, when rinsed in alcohol, the residue turned deep purple in color. It seemed to stain everything, including his fingers. Clearly this might be of commercial significance, so he continued his experiments at home but kept the results secret from Hofmann. Tests soon showed his new compound colored silk vividly and seemed to exhibit no signs of fading so, in August 1856, the 18-year-old student took out a patent on his new dye. He called it mauvine ($C_{26}H_{23}N_4$), and this historic moment marked the birth of the aniline-dye industry. Perkin created several other commercial compounds, including alizarin and coumarin, but his main enthusiasm was for his new purple dye. It became so widely popular that satirists spoke of "purple measles" overtaking the world of fashion.

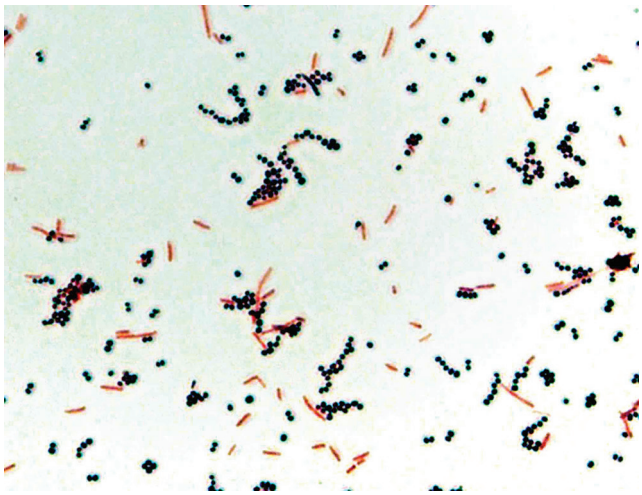
Europeans were also looking for improved stains. Saffron was such a brilliant dye that attempts were often made to create an artificial version and in Lyon,

France, a chemist François-Emmanuel Verguin was determined to synthesize a version he could market. He resolved to call it safranin, after the original saffron, though the dye he obtained in 1856 turned out to be a deep red color, rather than yellow. Although Verguin was disappointed by the result, the aniline stain he had accidentally created became one of the most popular in microscopy. Fuchsin was then created by Polish chemist Jakub Natanson in 1856, as the subject of his master's thesis, and crystal violet was synthesized by a French experimenter, Charles Lauth in 1861.

The English supremacy in producing aniline dyes was soon to be eclipsed by Germany. After working at a dye company in Manchester, England, the Polish-born chemist Heinrich Caro resolved to create new synthetic dyes and moved to Germany in 1861. He created eosin in 1874 and, rather than bestow on it a chemical term, he named it after his first girlfriend Eos. Caro created methylene blue in 1876 and malachite green the following year. The company he set up was the Badische Anilin-und Soda-Fabrik (the Baden Aniline and Soda Factory), and we still know it today as BASF. A range of German companies still familiar to us came into being as producers of their own versions of aniline dyes. Agfa gets its name from Aktiengesellschaft für Anilin Fabrikation, Corporation for Aniline Production, and so many others — Bayer, Geigy, Hoechst, and Sandoz — began by making aniline dyes 150 years ago.

One of the pioneers of using of these early microscopical stains was the Czech microscopist Jan Evangelista Purkyně. In 1837, using pre-achromatic compound microscopes, he identified the neurons in the cerebellum, which his name commemorates to this day as Purkinje cells. It was a remarkably diligent investigation, and today we recognize these neurons as among the largest cells in the brain. They have around 500 dendritic connections and emit curiously complex signals that may yet prove to be fundamental in studying how the brain functions.

The first microscopist to advocate the routine staining of sections was Joseph von Gerlach in Germany. He started using carmine in 1858, and it was in 1871 that Karl Weigert, a German-Jewish pathologist, began to use aniline stains in pathology. His cousin Paul Ehrlich is remembered for his introduction of staining in 1882, after attending a lecture on tuberculosis by Robert Koch. Using Caro's eosin as a counterstain for hematoxylin had been proposed by A. Wissowzky in 1876. The same technique is with us unaltered, to this day.



While working in the Berlin morgue in 1884, Hans Christian Gram devised the widely used staining method that still bears his name. In this micrograph, staphylococci retain the crystal violet coloration, while the Gram-negative *Pseudomonas* bacteria are stained red. We now know that these differences are due to the varying biochemical compositions of the bacterial cell wall.

GRAM AND POLYCHROME

Gram staining, a routine procedure in today's bacteriology laboratories, emerged from the experiments carried out by Hans Christian Gram, a Danish botanist and physician. Gram was the first person to recognize that macrocytes in a blood smear were diagnostic of pernicious anemia, and in 1884, based at the city morgue in Berlin, he devised his unique method of staining bacteria using crystal violet. This deep purple stain was never developed to color textiles but became popular with microscopists for the intense color it imparted to otherwise translucent specimens. Crystal violet was used as a bacterial stain, followed by flooding the slide with iodine solution, which acted as a mordant to make the dye less liable to fade with time. Gram showed that, if a stained preparation was then irrigated with alcohol, many kinds of bacteria would lose the deep blue stain and could be counterstained with safranin. In this way he divided bacteria into two groups, Gram-positive (dark blue) and Gram-negative (red) which he realized could help identify specific organisms.

The basic principle of the Gram stain centers on the ability of the bacterial cell wall to retain the crystal violet dye during solvent treatment. Gram-positive microorganisms have relatively thick cell walls with a peptidoglycan content of around 90%, which stains deeply with crystal violet and retains it when irrigated with alcohol. Gram-negative bacteria have thin cell

Malachowski and Romanowsky both tried blending methylene blue and eosin to give polychrome stains to emphasize specific components within the cell. Trial and error proved that old methylene blue solutions should be used, when the stain had been partially oxidized. These detailed studies of the malaria parasite developing in blood cells were published by Romanowsky in 1891.

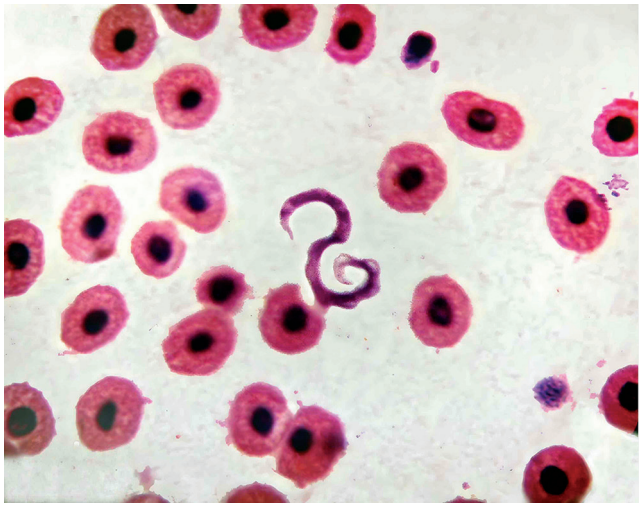
walls with little peptidoglycan but a high lipid content, and they do not retain the purple coloration, so the safranin counterstain makes them visible as bright red organisms in the finished smear. Gram thought his stain was merely an imperfect tool in identifying bacteria, but to this day we speak of Gram-positive and Gram-negative bacteria as part of the way we delineate them. The name of that unassuming Danish microbiologist remains part of our daily vocabulary in bacteriology — and the science clearly runs in the family. Gram's great-granddaughter, Lone Gram, is a professor at the Technical University of Denmark, where today she heads the Department of Bioengineering.

In 1891, the idea arose of using a mixture to provide polychrome staining. It was discovered by two scientists independently, Ernst Malachowski in Poland and Dmitri Leonidovich Romanowsky in Russia. To this day it is known as Romanowsky staining, though Malachowski deserves recognition. He was a successful and well-travelled physician, and because of his knowledge of English, Malachowski was chosen to act as guide to King George V when he visited Breslau. In 1890, Malachowski demonstrated treating methylene blue with borax and showed that it stained blood smears containing the malaria parasite *Plasmodium*. The structure within the *Plasmodium* cells showed up with dramatic clarity. Romanowsky was working in Russia at the same time, and in 1891, he realized that oxidized methylene blue, left to mature as a fine wine might age, produced this effect. A mixture of the matured methylene blue and eosin provided a spectrum of colors that allowed the microscopist to identify a range of substructures within cells. And thus polychrome staining was born.

The principle was adopted by many microscopists, and adapted by several. In 1902, new versions of the technique were announced. Gustav Giemsa added glycerol and blue dyes to the mixture and created an excellent staining protocol for the rapid screening for malaria, trypanosomes, and *Chlamydia*. He was to become a devout Nazi during the 1930s. At the same time, Sir William Boog Leishman, a Scottish Lieutenant-General in the British Army, invented his own version of the stain to identify the causative agent of kala-azar; the organism still bears the name *Leishmania*. James Homer Wright, a pathologist at Massachusetts General Hospital, perfected his version, which proved to be of particular use in the differential staining of white blood cells. In today's laboratories, Wright, Giemsa, and Leishman stains are routinely used, and all are well over a century old.

It seems curious that a dye industry should emerge from a quest for medicine. In fact, many of these compounds, familiar to us as microscopical stains, also have strongly antimicrobial propensities. Safranin and crystal violet were recently tested as antibacterials and proved to be more effective than iodine solution against drug-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Methemoglobinemia can successfully be treated with methylene blue, which is also an effective antidote to cyanide poisoning. Malachite green is widely used in aquaculture to control outbreaks of the oomycete fungus *Saprolegnia* in fish farms and can eliminate the parasitic ciliate *Ichthyophthirius multifiliis* in aquariums. So when the young Perkin was seeking a cure when he accidentally stumbled across aniline dyes, he was right all along; many of these brilliantly colored compounds really can cure diseases.

Although hematoxylin and eosin remain the mainstay of present-day histological staining, special stains (we still call them that) first emerged in the 19th century. These are stains that identify specific components within tissue samples, and the first to be introduced was Prussian blue, $Fe_7(CN)_{18}$. It was introduced as a specific stain for iron in 1867 by a pathologist in Germany, Max Perls, though he was not the first to observe the reaction. That insight belongs to Rudolf Virchow, the German physician widely regarded as the father of pathology. Virchow published over 2,000 papers, and coined terms including spina bifida, and parenchyma. He was the first to record amyloid, currently a focus of research in neurodegenerative diseases, and he used to proclaim the doctrine of *omnis cellula e cellula* (from cells come all cells), a phrase he plagiarized from the Polish embryologist Robert Remak. It was Virchow who first described brown pigment deposition in cases of hemochromatosis in 1847, and he noted that the deposits reacted specifically with Prussian blue. It fell to Perls, a young scientist working at Königsberg, who formalized its use as a selective staining procedure. He described it as identifying pachymeningitis hämorrhagica, a stain widely used today.



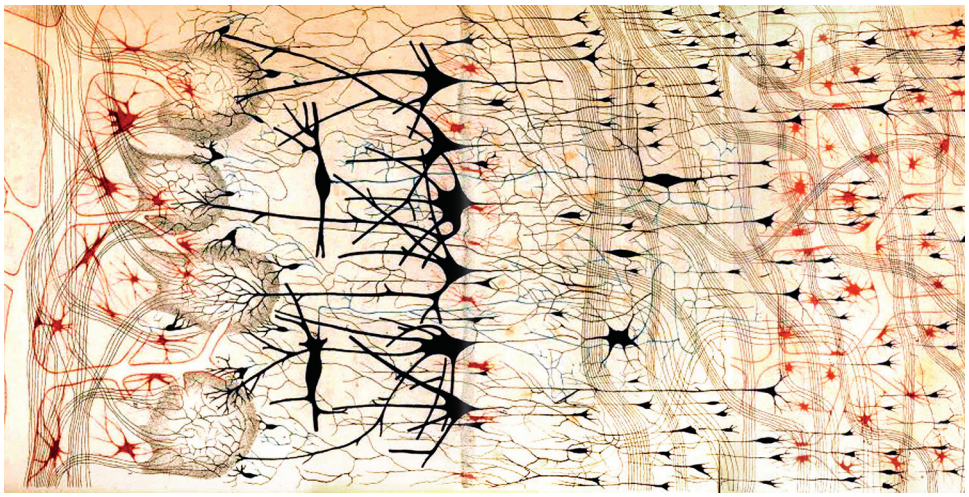
When I was 20, a friend told me of fish in a nearby lake that were acting strangely. Under the microscope I could observe living trypanosomes in their blood, and preparations stained with Gustav Giemsa's invention of 1902 revealed the organisms clearly. The mixture of methylene blue and eosin with Azure B brings out the erythrocyte nuclei clearly, as well as displaying the parasite.

GOLGI'S "BLACK REACTION"

The study of nerves remained problematic. The earliest microscopists tended to view nerves as tubes, along which fluids might pass (I discussed this at length in 2007; see "Enlightening Neuroscience: Microscopes and Microscopy in the Eighteenth Century," https://doi.org/10.1007/978-0-387-70967-3_3). Nervous tissue, and notably the brain, was seen as an amorphous greyish mass with little substructure. Aniline dyes stained the specimens well enough, but the result was a confusing mass of fibers and seemingly amorphous tissue. This convention was overthrown one day in 1872, when Camillo Golgi was researching under Giulio Bizzozero in the Institute of General Pathology at the University of Pavia, Italy. His interest was the structure of the brain, and Golgi knew that brain tissue conventionally stained would not reveal its secrets.

The method in use was to stain a small block of tissue with carmine or hematoxylin. The soft specimen was perfused with chromic acid or potassium dichromate, which fixed and hardened the tissue, allowing microscopists to tease out individual neurons with fine needles from the semi-solid specimen. It was not a success. Each neuron is endowed with numerous fine side branches, the dendrites that carry impulses into the cell, and the axon which transmits the resulting signals away from the neuron. These were inevitably broken off by the micromanipulation that was necessary to separate the body of the neuron from the rest of the specimen.

Instead of conventional staining, Golgi decided to try perfusing tissue samples with metallic salts in the hope of increasing the visibility of its microscopic structure. He experimented in his spare time, working by candlelight, in the kitchen. He had tried salts of mercury and of gold, without success, and one evening decided to try silver nitrate. He soaked small samples of brain in 2.5% aqueous potassium dichromate for a few weeks. The specimens were then transferred to 1% silver nitrate solution and left until they had turned the color of coal. Golgi described the technique as *la reazione nera* (the black reaction). He then dehydrated the tissue samples by passing them through increasing strengths of ethanol, and then through mixtures of ethanol and turpentine before perfusing them with resin. He was astonished to see nerve cells picked out in black against a pale golden background. Golgi was looking at intact, entire neurons for the first time in history. He later cut sections at 100 μm (one-tenth of a millimeter), which is 10 times the thickness of conventional tissue sections, but which allowed Golgi to trace the rise and fall of dendrites as they wandered through the surrounding tissues. The results were vivid and entirely unexpected. Not only could Golgi clearly observe neurons picked out as intense black against a



In his quest for a metal compound that could stain neurons, the Italian microscopist Camillo Golgi happened upon silver nitrate. This study of canine olfactory innervation was published by Golgi in 1875. It is extraordinarily detailed and meticulously observed, though his silver stain detects very few of the neurons in a specimen — and still nobody knows why.

golden background, but the axons and dendrites were seen as a fine, dark tracery of undulating fibers. Gone was the notion of brain tissue as formless and essentially homogeneous; it was a community of stunningly beautiful cells in a network of immense complexity.

In August 1873, Golgi published his results in the *Gazzetta Medica Italiani* under the title: *Sulla struttura della sostanza grigia del cervello* ("On the structure of the gray substance of the cerebrum"). For a decade he expanded his studies, eventually publishing all his findings in 1885 as a book, *Sulla Fina Anatomia degli Organi Centrali del Sistema Nervoso* ("On the Fine Anatomy of the Central Organs of the Nervous System") published by Ulrico Hoepli in Milan. Golgi's new techniques were adopted by Swiss microscopists, including Eugen Bleuler and Albert von Kölliker, both of whom extolled its merits, and word began slowly to spread about this revolutionary new technique.

NEURON BREAKTHROUGH

Two years later, Golgi's work came to the attention of the Spanish physician and microscopist Santiago Ramón y Cajal, who had embarked on studying the fine structure of tissues. On a visit to Madrid, he was shown sections stained by the Golgi method by Don Luis Simarro Lacabra, a psychiatrist who hoped to learn more about the brain's internal structure. Cajal was astonished at the clarity with which neurons could be seen and wrote to compare the vividness of the Golgi stain with the "tangled thicket where sight might stare and grope ever fruitlessly" in sections conventionally stained with "carmine or logwood." He experimented with Golgi's original method, modifying it to optimize the results and then embarked upon a unique study of the cerebellum and retina. It was Cajal who realized that the synapses are where extensions of brain cells meet, yet do not touch. His work provided the impetus needed to launch the notion that the brain was not a vast and confused syncytium but comprised a community of discrete cells. He studied the neurons, not as we do now, by cutting fine sections perhaps 5–10 µm thick, but by cutting thicker slices up to 100 µm, just as Golgi had pioneered. Cajal worked by following the delicate tracery of fine fibers that emanate from each neuron, changing focus to follow each thread as it wove its way through the tissues. In this way, he could draw each neuron in its entirety, whereas a conventional section would never show the complete cell. He could trace the rising and falling paths of each fiber, which would vanish from view in a thin section, and draw them painstakingly with ink on paper. He also observed the minute dendritic spines that appeared along so many of the fibers, like thorns on the stem of a rambling rose. Cajal's colleague and co-worker Wilhelm von Waldeyer-Hartz later named the cells neurons, and Cajal's research gave rise to the neuron theory that underpins today's understanding of the brain.

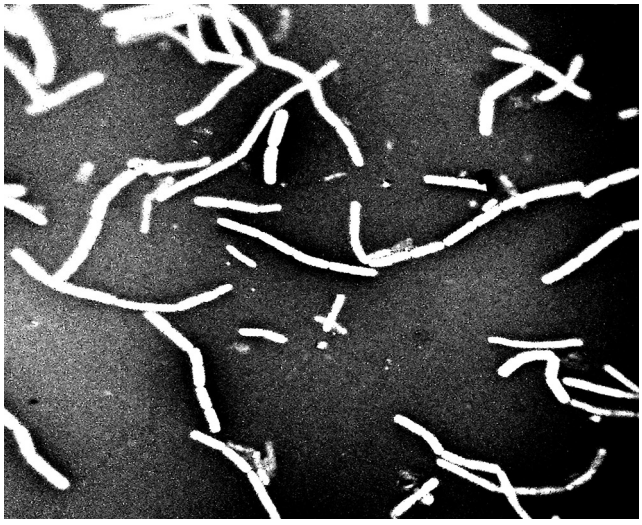
Using digital technology, we can try to emulate Cajal's results by linking stacked images, but nobody has approached the diligence with which Cajal manually recorded his meticulous observations. Golgi's technique was so revolutionary that he was proposed for a Nobel Prize every year from 1901–1905, though without success. When Cajal's work appeared, so revealing were the new findings that he and Golgi were successfully nominated for a joint award for Physiology or Medicine in 1906. This was the first time that the prize had been awarded jointly, and Cajal was the first Spaniard to receive a Nobel Prize.

His drawings remain unsurpassed to this day — yet there remains a puzzle nobody can solve. If the Golgi reaction stained every neuron, the observer would be confused by the endless intricacies of the intertwining dendrites. In fact, only around 10% of the neurons in a sample take up the stain, and still nobody knows why. The proportion that become stained makes life much easier for the microscopist. It may be that some cells are impregnable to the reagents, or that neurons need to be in a specific physiological state of activity when harvested. But whatever the reason, the proportion of neurons that stain is just what we need to observe them individually. It's a puzzle, and one that allowed Golgi and Cajal to claim their Nobel Prize. If the tissues they studied behaved like any other specimen, they would never have seen what they did.

SEEN LIKE THE STARS

Staining specimens with brightly colored dyes is not the only way to endow them with greater visibility. They can also be revealed, not by what color they become but when their surroundings provide them with greater contrast. This gave rise to negative staining, when the background to a preparation is darkened, so that microscopic organisms — bacteria, and even flagella — can be visualized as clearly as stars in the sky. The technique emerged during the Victorian era. Because it's so simple to use and so elementary in its conception, we cannot see precisely who first discovered it. Microscopists using the technique may have considered it too obvious to be worth describing in detail. All you do is mix a droplet of Indian Ink with a tiny sample of a suspension containing microorganisms, and let it dry on the slide. That's it. It doesn't need a coverslip and involves no mounting methodology. It becomes a black blob, like paint, on the slide.

But it is a blob that contains microbes, and they dry to show as transparent organisms with the black ink lying around them. We have seen that cells bear a negative charge, same as the components of Indian ink, which results in mutual repulsion. Rather than absorbing the black dye, the living cells repel it and so, as the ink forms an impenetrable black layer, like varnish on the slide, any living cells transmit light from the substage, so they shine brightly in the eyepiece. Bacteria (like *Spirillum*) show up vividly using negative staining, and it is a technique widely used to differentially diagnose *Cryptococcus*, cells of which are surrounded by a transparent capsule. In cases of cryptococcal meningitis, negative staining with Indian ink provides a rapid and reliable test that can distinguish the pathogenic yeast from other organisms.

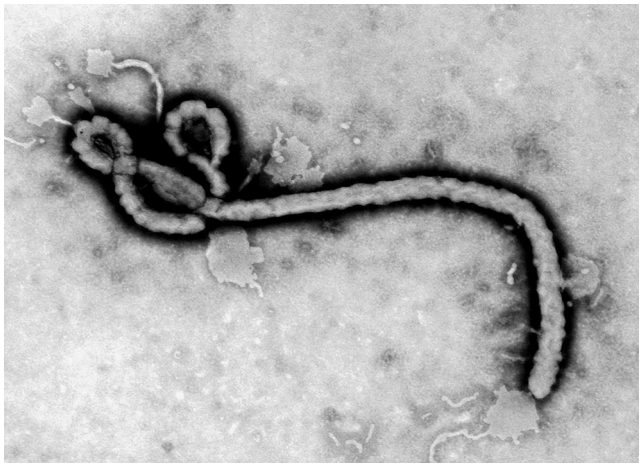


Indian ink dries to form an opaque film on glass. If bacteria, like these bacilli, are first mixed into the droplet, they show up as transparent outlines against a dark background. This negative staining was first used by Victorian microscopists, though the date of discovery is unknown. The technique can also reveal flagella and the diagnostic capsule surrounding *Cryptococcus* cells.

Indian ink was discovered in ancient China. The earliest records reveal that wicks fueled by oils and lard were lit to provide the black, amorphous carbon soot from which the ink was made. In time, it was also obtained by burning softwood. Combustion was in a chamber with a small chimney outlet; the soot was removed when the chamber had cooled, by being brushed off with a goose quill. The fine soot powder was then blended with animal glue and allowed to dry into a small cake or stick. A similar process was subsequently developed and copied in India, where the product was known as *masi*.

Around the 1660s, coincidentally when microscopy was beginning to emerge, burgeoning trade between Britain and India brought this durable product to England for the first time, and this is when it became known as Indian ink. Microscopists used it to mark the rings around circular coverslips, when glass slides replaced bone and ivory sliders around 1840. Negative staining emerged around the same time.

Its origins may be ancient, but Indian ink is widely used in today's laboratories, quite apart from providing the inky-black background for negative staining. Dilute suspensions are used to demonstrate the ingestion of particles by feeding cells (like phagocytes). Microscopists also use it to draw small circles on slides to indicate the precise position of an object of interest.



The Ebola virus has often hit the headlines, and this remarkable image seems to typify current research — but it is almost 50 years old. Frederick A. Murphy, electron microscopist at the Centers for Disease Control in Atlanta, photographed this on Oct. 13, 1976. He used PTA negative staining to reveal what he called the "dark beauty" of the virion.

I'm told that pathologists use Indian ink to mark the edges of excised tumors, prior to microscopical examination, and it has been injected into capillaries to disclose the delineation of the smallest blood vessels in cleared tissue sections.

My late friend, the expansively named Noel Joseph Terence Montgomery Needham at Cambridge University, investigated Indian ink for his monumental series *Science and Civilization* in China. He began publishing this prodigious work in 1954, and the last time we met in 1994, nearing the end of his life, Needham was still busily correcting page proofs. The results are currently available in a series of 27 hardbound books, and more are still in the pipeline. Needham investigated the prehistory of Chinese innovations, and in volume 5 of his great work, (part 1, page 23; published in 1985) he found that this enduring ink had been invented by the Chinese some 3,000 years ago. Characters written with black ink on bones are known from about 1,000 B.C., during the Shang dynasty. Manufacturing the ink did not emerge in India for another 1,500 years.

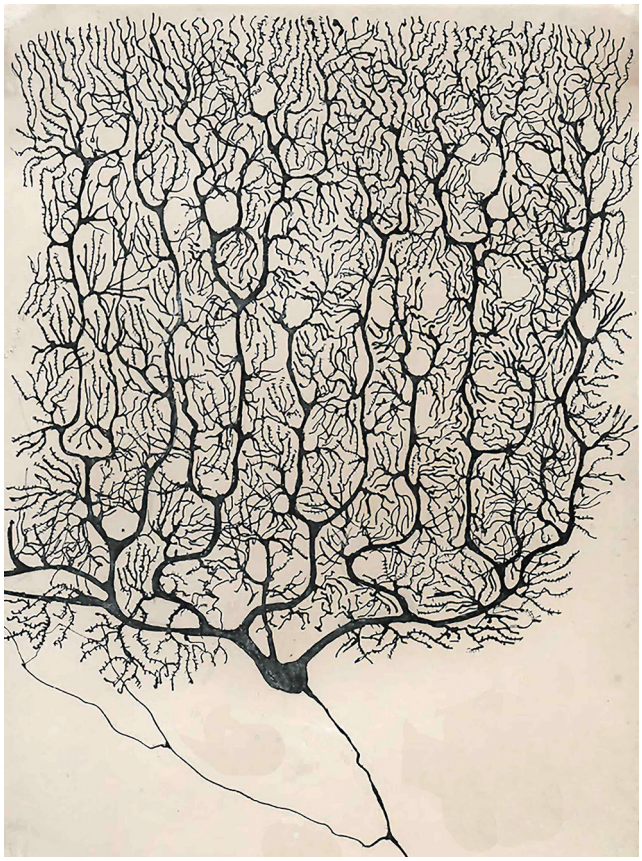
The principle of negative staining was to be revolutionary in transmission electron microscopy (TEM). It was developed by a Canadian researcher named Robert Horne at the Cavendish laboratory in Cambridge, where I carry out my scanning electron microscopy today (indeed, Horne's original microscope is still on display upstairs). I met Horne only once, though he studied under Vernon Ellis Cosslett, an extraordinarily gifted electron microscope pioneer whom I knew and visited at his home. These were insightful and broadly educated scientists, who revolutionized the field. Between them they introduced negative staining techniques to electron microscopy, which allowed us to study viruses in greater detail than ever before.

Bacteriophages were the first viruses to be observed by Ernst Ruska ("Germ Versus Germ," *The Microscope*, 69:4, pp 163–174, 2022; doi.org/10.59082/EXKW5818), and the excitement of seeing a virus particle for the first time triggered a wave of interest in methods of improving image quality under TEM. Horne worked with Sydney Brenner, a remarkable South African biologist who had been accepted into the University of the Witwatersrand aged just 15. Brenner later spent 20 years at the Laboratory of Molecular Biology at Cambridge, before joining the Salk Institute in California in 1976.

Horne and Brenner recognized that an electron-dense fluid could bring out detail in viruses, which would seem like bright bodies against a dark background — exactly like the negative staining of bacteria with Indian ink. Their stain of choice proved to be phosphotungstic acid (PTA, $H_3PW_{12}O_{40}$), which gave them an electron-dense background against which to view their viruses. The results were vivid and unforgettable; polio virions no more than 20 nm in diameter stood out like snooker balls on grey baize. The famous image of the Ebola virus that you see in all the articles was stained with PTA in this way. That image was captured by Frederick A. Murphy at the Centers for Disease Control (CDC) back in 1976. Other electron-dense negative stains have since been added to the list. We now use osmium tetroxide, ammonium molybdate, and uranyl acetate, among others.

Surprisingly, PTA has also found its place in light microscopy. Mixed with phosphomolybdic acid, it can react to form a blend of oxides of tungsten and molybdenum. The staining solution is blue, with maximum absorption around 750 nm, and has been harnessed in the Folin-Ciocalteu Method for phenolic compounds. PTA can also be mixed with hematoxylin and used as a muscle stain with a special affinity for mitochondria. The hematin in the tissue sections binds with PTA to form a blue lake, with the excess PTA staining collagen brown. So the time-honored principle of negative staining has given rise to a revolution in the electron microscopy of viruses, and thereafter re-entered the world of the light microscope as a valuable tissue stain.

Scientists working with electrophoresis rely on Indian ink to visualize discrete proteins during performing Western blot tests. In this procedure, protein molecules are transferred to a membrane comprised of cellulose nitrate, popularly known as nitrocellulose.



Spanish microscopist Santiago Ramón y Cajal treated brain sections with Golgi's silver stain but took it one step further. Golgi drew neurons, so Cajal set out to meticulously trace each dendrite to visualize the complexity of a single brain cell. In 1904, this Purkinje cell from the human cerebellum inspired him to see neurons as "butterflies of the soul."

Scientists at the Stevens Neuroimaging and Informatics Institute at the University of Southern California have developed an eye-catching means to capture the way individual neurons are aligned in the medial prefrontal cortex of the brain. These facilities cost millions, yet they cannot trace dendrites as clearly as Cajal did over a century ago, working at home by lamplight. Modern research is different but not always better.

Membranes of this substance are widely used as a substrate for the culture of living human cells, and conventional microscope slides coated with a thin cellulose nitrate layer, known as nitrocellulose slides, are widely used in specialist laboratories.

This too has historical roots; cellulose nitrate was the first plastic to be discovered — by accident. Henri Braconnot, a French pharmacist, was an ardent experimenter. One of his revelations was that cell walls in fungi, like the exoskeleton of arthropods (and unlike the cell walls of plants), are composed of chitin. In 1832, he spilled some nitric acid and, as was always done in emergencies at the time, threw sawdust on it to absorb the liquid. Sawdust was popular for centuries. When I was young, a butcher's store always had a dusting of sawdust on the floor to absorb any drips of blood. To Braconnot's surprise, the nitric acid reacted strongly with the sawdust to produce a dry, solid residue. This caught his attention, and he conducted further trials, before announcing that he had discovered a new material he called xyloidine. It proved to be unstable and highly inflammable, though this was the first synthesized plastic in history. Six years later, another French experimenter, Théophile-Jules Pelouze, announced much the same discovery, this time because of nitric acid reacting with waste paper and card. Pelouze named his version nitramidine. He is the man who taught Alfred Nobel. How curious that nitramidine was considered a likely candidate for an industrial explosive, and Nobel would make his name (and fortune) by inventing dynamite.

AN EXPLOSIVE IMPACT

Even though both experimenters announced their discoveries and gave the resulting products trade names, little more was heard of either until 1845, when a German chemist, Christian Friedrich Schönbein, was

experimenting in the kitchen at home in Basel, Switzerland. He too accidentally upset some concentrated nitric acid and seized his wife's cotton apron hanging nearby to mop it up. He hung up the damp apron to dry by the stove. Not only did it dry out, but it flashed into flame. The cellulose had reacted with the concentrated acid to produce cellulose nitrate. Schönbein had discovered guncotton. He realized it could make the ideal explosive instead of gunpowder. Gunpowder rapidly deflagrates, rather than suddenly exploding, and it creates smoke. Guncotton was easier to handle, and produced a quick, clean explosion. Schönbein sent samples to colleagues around Europe, all of whom thought it a remarkable invention, though he said nothing of the method of preparation until he had safely secured a patent.

Schönbein knew Rudolf Christian Böttger, a German chemist at the University of Frankfurt, who had made exactly the same discovery in the same year and for a time the two cooperated. They were not alone. Precisely the same observation was made in Germany at Braunschweig University by F.J. Otto, who wasted no time and published his results in 1846. By this time an English company, John Hall & Sons Co., had bought out the patent rights from Schönbein and, early in 1847, they opened an extensive guncotton factory in Faversham, England. The product was popular, and production went ahead smoothly until (just as Schönbein had discovered) there was a huge spontaneous explosion that destroyed the building and killed most of the workers. The same disaster happened at a new guncotton factory in Bouchet, near Paris, France. Clearly, it was a product not to be trusted. It was Wilhelm Freiherr Lenk von Wolfsberg, commissioned in 1849 by the Austrian army to find a safe way to manufacture guncotton, who took two years to solve the problem. A factory was set up in Hirtenberg, Austria, to produce guncotton. The factory didn't explode. They went on to manufacture trinitrocellulose $C_{12}H_7(3NO_4)O_{10}$. The company Hirtenberger AG is still there to this day, and it has become one of the world's largest exporters of ammunition.

Cellulose nitrate became widely known as nitrocellulose, and other applications were soon found for it. It proved to be readily soluble in organic solvents like acetone, and was used to make high-gloss lacquer for furniture. In 1855, Alexander Parkes, an inventor in Birmingham, England, found cellulose nitrate reacted with camphor to produce a plastic he named after himself — Parkesine. It was never successful, but in 1870, the American New York inventor John Wesley Hyatt announced the production of cellulose nitrate as Celluloid, which he used to make a range of domestic items from hair combs to utensils, and provided the base for the first movie films. It was even spun into the first artificial fiber in 1890. Cellulose nitrate is used today to hold staples together in strips, and it can be used to take peels from fossiliferous rocks etched with acid. It provides the shiny surface on playing cards and a guitar plectrum is made of cellulose nitrate (yes, they are highly inflammable).

It is the affinity of cellulose nitrate for amino acids that makes it the ideal substrate for the mobilization of proteins in Western blot tests and DNA in Northern and Southern blots, and it underpins atomic force microscopy. Cellulose nitrate creates membrane filters, used to separate fine particulates from suspension. When dissolved in a mixture of ethanol and ether, it forms a syrupy substance you all know as collodion. Films on glass found application in the wet collodion photographic process, which eclipsed the widely used Daguerreotype and served to popularize photography. Collodion has been widely promoted as artificial skin, used to dress wounds, but has other uses. It holds preparations of salicylic acid in place in commercial wart remover, and in microscopy a single drop on a snowflake creates a cast that allows the shape of the flake to be studied under the microscope, long after it has melted.

We forget its inflammable nature to our peril. On Aug. 12, 2015, cellulose nitrate triggered a massive explosion in the port of Tianjin, China, which killed 173 people, and wounded 1,000 more. Schönbein watched his wife's apron explode into flame in 1845, and the same phenomenon is lying in wait today.

BALSAM, TRIED-AND-TRUE

Then there is the saga of Canada balsam, one of the most important components in the history of microscopy. Look in a reference collection of slides; those with a mountant of a golden hue are the specimens mounted in Canada balsam. The glass-clear slides have specimens probably mounted in a modern mounting medium, often based on a viscous solution of styrene. Most online accounts of slide mounting recommend mountants made with glycerol, since the specimens do not require dehydration, and a slide is much simpler to prepare. However, these preparations are delicate, and the coverslip can easily be wiped away. The serious microscopist will prefer a permanent preparation that will last a century or more and can be polished with tissue. This is where Canada balsam came in.

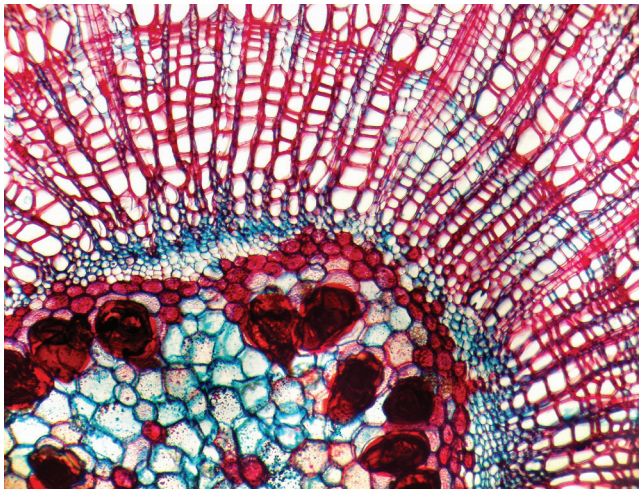
This classic mountant is a treacly resin from a Ca-

nadian gymnosperm, the balsam fir *Abies balsamea*. The sticky exudate hardens quickly to form a resistant varnish. When first used by microscopists, a droplet of the hardened resin was melted, and an insect could then be dropped into place and suitably positioned with a dissecting needle. A coverslip was gently lowered onto the droplet which would spread by the weight of the glass coverslip (and capillary action). Before long, solid Canada balsam was being dissolved in turpentine or xylene to form a viscous mountant that could be applied from a thin glass rod and allowed to dry. Solutions of Canada balsam became the mainstay of permanent preparations in the 1840s and many microscopists prefer to use it to this day. The refractive index of the crown glass from which slides and coverslips are manufactured is 1.520, and that of Canada balsam lies between 1.520 and 1.540, making it a good match.

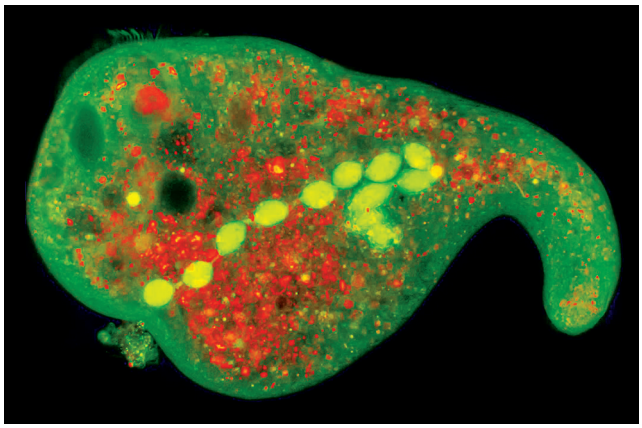
It remains fashionable. Numount is one of the modern alternatives and is still promoted as a "Canada balsam substitute." Enthusiasts will claim that the modern mountants are sometimes liable to detach from the slide or crystallize; they insist that Canada balsam never lets you down. It does, though. This is an ideal mountant in that it never forms crystals and doesn't become detached from the glass — but sometimes it darkens with age. This is said to be a property of impurities (I have looked at slides from over 150 years ago that remain as clear as the day they were made) and devotees won't ever relinquish Canada balsam as the only mountant worthy of the name.

Its use dates back thousands of years before microscopy. Chippewa medicinal traditions feature Canada balsam as an analgesic. It was also applied to minor abrasions and was used to treat cold sores. When the trees were first exploited by Europeans, the resin was widely used as wood varnish on fine furniture, but because it often tended to darken with age, its use by furniture makers was discontinued. The first account of Canada balsam as a microscope mountant was published in 1835 by Andrew Pritchard of London, in his book *A List of 2,000 Microscopic Objects*. In the opening section on the mounting of specimens, Pritchard reports a method of mounting that he has just started using "with great success." On page 6 of his book he states: "It consists in immersing the object in Canada balsam or varnish, and pressing it between two slips of glass so as to exclude the air-bubbles." That is the first mention of a method close to current practice in the literature.

It is not, however, entirely without precedent. Between 1739–1744 a microscopist in London named William Hewson made a large number of preparations of teased and flattened tissue specimens for the pioneering anatomical investigator John Hunter. They were spread on small glass slides, dried, and then coated with varnish. The collection resides to this day at London's Hunterian Museum, and they were described in detail for the Royal College of Surgeons by Jessie Dobson in 1960. Dobson found that many were still in viewable condition. The earliest date we have for mounting microscope specimens in varnish is 1739 — and could it have been Canada balsam? Mounting in Canada balsam between slips of glass was popular in 1835, and the Royal Microscopical Society



Stained with safranin and light green, this transverse section of *Ribes* stem clearly shows the details of plant histology. We imagine that we are at the forefront of science when we work on microscopical specimens, but most of the routine stains and reagents we use every day were introduced centuries ago, and some date back for thousands of years.



Fluorescent stains have become more widely used in recent decades. Dyes that target specific cellular components and fluoresce under UV were recognized in 1845 by Fredrick W. Herschel, and fluorescence microscopy became established around 1942. This example by Canadian microscopist Robert Berdan allows us to study the structure of *Stentor* and its beaded meganucleus.

introduced the standardized slide in 1840, where specimens would be mounted beneath a coverslip. The RMS Standard slide, measuring 3 × 1 in., is used throughout the world to this day, even though it is now defined as 75 × 25 mm.

Formalin, a 40% aqueous solution of formaldehyde, became globally popular as the universal preservative for tissue samples throughout the 20th century, though now it's being banned. It was originally discovered by accident. Russian chemist Alexander Mikhailovich Butlerov (the man who first identified double bonds in chemical structure) recorded it in 1859 while trying to synthesize methylene glycol. But Butlerov had the formula wrong. He thought it was $C_4H_4O_4$, which we now call fumaric acid, though he called it methylene dioxide. It fell to August Wilhelm von Hofmann, the German chemist who had pioneered the study of aniline, to correctly identify the molecule as CH_2O and invent a manufacturing process in which vaporized methanol was passed over a hot platinum wire. He published his findings in 1867, and by the turn of the century formaldehyde was in commercial production. It found uses as an embalming fluid, preservative and disinfectant, but then Bakelite plastic was discovered by the Belgian entrepreneur Leo Bakeland in a barn behind his home in Yonkers, New York. Bakelite was made by reacting phenol with formaldehyde to produce the famous thermosetting resin — polyoxybenzylmethylenglycolanhydride. This has since been used to make so many items of our daily lives, from phones and switches to TV sets and the cap of an automotive distributor. The global production of formaldehyde gave impetus to its use in other areas, and for the entire 20th century it was the most useful tissue preservative we used in the laboratory. Then it was found to be a carcinogen, so its use was progressively restricted. After a century of prominence, formaldehyde was relegated to the danger list — but only after opening so many avenues for investigation while it lasted.

EARLY SECTION CUTTING

Finally, what of section cutting? The first book on microscopy was Robert Hooke's great work *Micrographia* of 1665. Among the illustrations was an image of a section of cork he had cut by hand using a shaving razor. It's impressive — and thin. Commentators have always insisted that the methods used by the pioneers were crude. Writing on section cutting in 1978, Brian Bracegirdle asserted that "no preparations from the seventeenth century existed" adding that those dry mounts would have been prepared "with little finesse." In a BBC program on the cell in 2009, presenter Adam Rutherford said: "botanists had been eagerly tearing up plants to study their anatomy." Tearing? Those pioneers were cleverer than we think. Nobody accepted that the first researchers were capable and diligent individuals, and it was always assumed that Hooke's engraving was of an idealized section. Surely no 17th century pioneer could cut sections of such precision. But Leeuwenhoek had been influenced by Hooke's book on a visit to London and had been inspired to copy his example. Leeuwenhoek's own cork sections were equally as fine as those that Hooke had portrayed. They were as good as an experienced microscopist might make today. It was Leeuwenhoek who introduced the idea of serial sections, a technique still widely used, as he carefully cut a cotton seed into 30 consecutive slices. He also cut sections of dried bovine optic nerve to show its appearance in transverse section. His slices were cut with a razor, using air-dried nerve specimens. He had no other way to do it. To cut fine sections of soft, yielding animal material you would need to embed the specimen in something to hold it firm.

Paraffin wax from candles proved to be the answer. It was first used as an embedding medium by Eduard Fenz in Vienna, Austria, during the 1840s. It was adopted by the Hungarian pathologist Salomon Stricker in 1864 and popularized in 1868 through the work of Wilhelm His Sr., a Swiss anatomist. By 1869, Swiss microbiologist Edwin Klebs had shown that candle wax could be used to embed specimens by passing them through dehydration in alcohol and then clearing them in turpentine. And today? The same wax is used in laboratories around the world for routine section cutting in histology. Others have been tried (like soya wax), but nothing can match the paraffin wax first used to make candles in Derbyshire, England, in 1848.

When you look around your laboratory, it is easy to be overcome by the sheer newness of the equipment: the bleeping computers, the blinking LED displays, and the hum of electricity. But the stains? The reagents? The techniques that modern microscopists utilize every day? Some date back thousands of years, and (at the very least) over a century. Most of the procedures we use are those devised by our Victorian antecedents and many popular staining systems — Giemsa, Leishman, Wright, Gram — commemorate ingenious scientists we should think of with respect. They laid down protocols for the future, and we still follow them. The history of microscopy is not some remote scholarly subject — it's around us every day. ■