Microscopy's Bright Side

Fluorescent dyes add color and sensitivity to biologists' perception of cells

BY JULIE ANN MILLER

This is the first of three articles on fluorescence techniques. See next week for fluorescence techniques' applications to geology and spectroscopy.

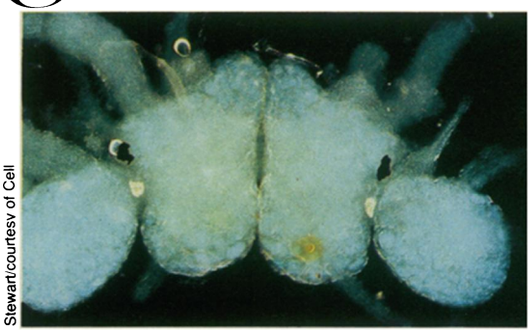
With all the dazzle of a neon sign, fluorescent dyes are lighting up biological microscopy. A nerve cell glows a brilliant yellow and connected cells give off a paler glimmer. The skeleton of a laboratory-grown cell radiates bright green and cell surface components dot a dark field with red. Improved techniques are allowing more and more scientists to answer questions simply by using their eyes.

Scientists open their eyes wide and sit up straight in a seminar when a lively, colorful micrograph replaces more mundane tables and graphs on a projection screen. But there's more to these techniques than eye-popping color.

Finding the proverbial needle in a haystack analogy, a fluorescence technique would be of limited use if the needle had to be labeled before it was accidently lost, or had to be found first in order to be labeled. But consider the value of a searching device that would go to the needle, attach and then glow.

The search device in biological experiments is usually an antibody molecule equipped with a fluorescent dye. In the most direct method, the researcher takes a pure sample of the target material and uses rabbits or, more recently, laboratory-grown cells to produce the required antibody. The researcher then chemically must link the fluorescent label to the antibody.

An indirect or "sandwich" technique is more commonly used than the direct approach, because it is simpler to carry out and can be even more sensitive. In the indirect procedure, one antibody chemically linked with a fluorescent tag is used to light up a wide variety of targets. The sequence of steps is, first, a nonfluorescent antibody is made to attach to the target molecule, and second, a fluorescently labeled antibody binds to the first antibody. For example, when Joseph H. Neale and Solomon H. Snyder wanted to visualize the nerve cells that contain the opioid peptide enkephalins (SN: 8/12/78, p. 103), they exposed the tissue sample to rabbit antibodies that bind to enkephalins and next to fluorescently tagged goat antibodies that attach to rabbit antibodies. They then could observe the green fluorescent glow in nerve cells with a wide variety of sizes and shapes. The greater sensitivity of the indirect technique re-



Stewart/courtesy of Cell

New dye, Lucifer Yellow (left), fluoresces more brightly than dye formerly used.

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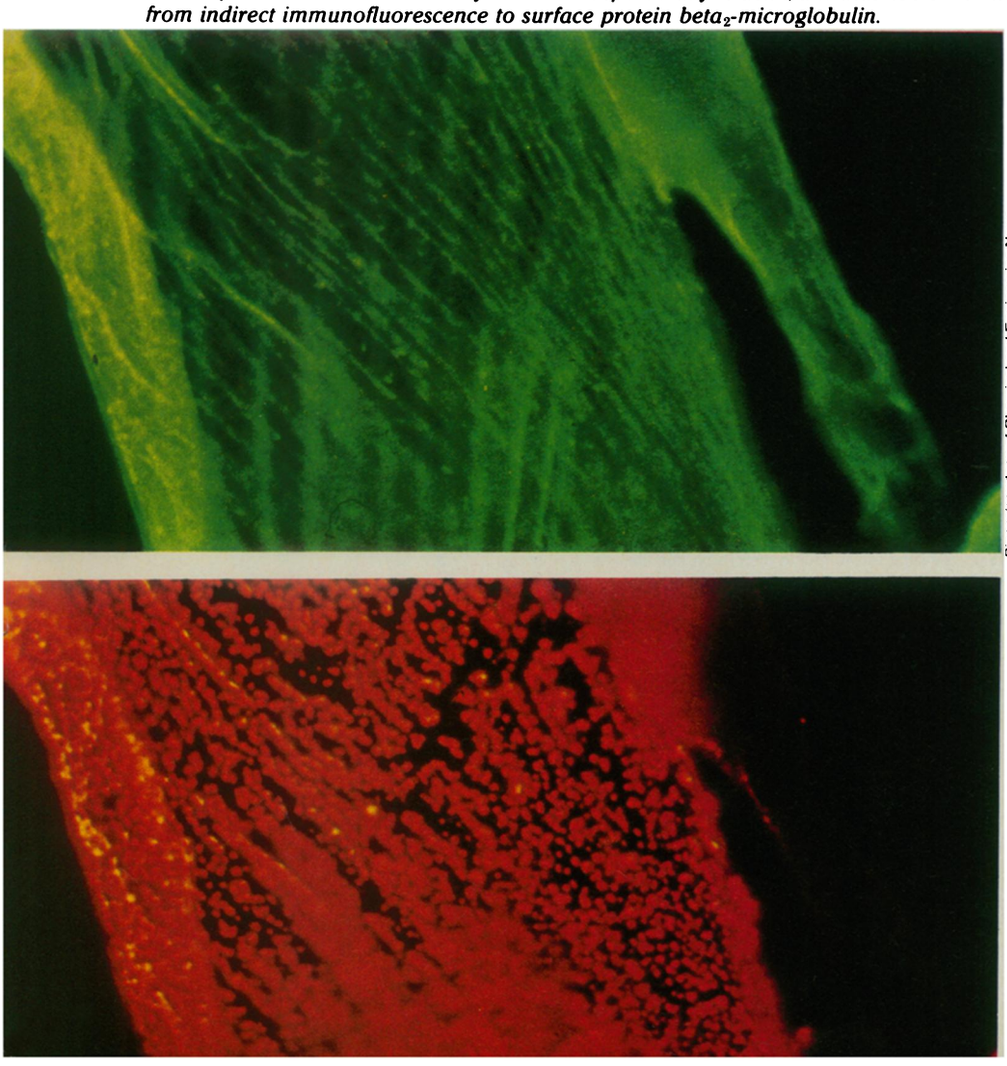
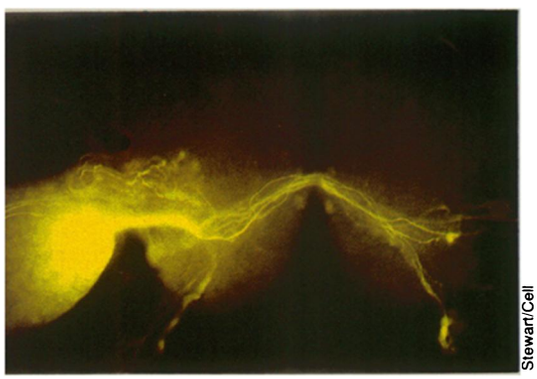
Simultaneous stain with two dyes reveals fibers inside cell lined up under clustered surface proteins. Green is from a dye that binds specifically to actin, and thus fibers. Red is from indirect immunofluorescence to surface protein beta$_2$-microglobulin.



Singer/courtesy of Chemical and Engineering News

sults because more than one antibody can react with the same target molecule. Thus the extra antibody step causes several times as many fluorescent molecules to attach, compared to the direct technique.

The antibody fluorescence technique has thrown light on three major research areas. One is the study of the fibers, filaments and tubules that act as the skeleton (and perhaps muscle) of cells. For example, Mary Osborn and Klaus Weber of the Max Planck Institute in Göttingen have visualized quite different web patterns for the three major intracellular fiber systems. Using the indirect immunofluorescence technique with antibodies to each type of subcellular fiber, they found that the microfilaments tend to run in parallel through a rat kangaroo's kidney cell, the microtubules radiate from the nucleus toward the cell surface and the intermediate fibers form a wavy, intermingled design (SN: 10/15/77, p. 250).

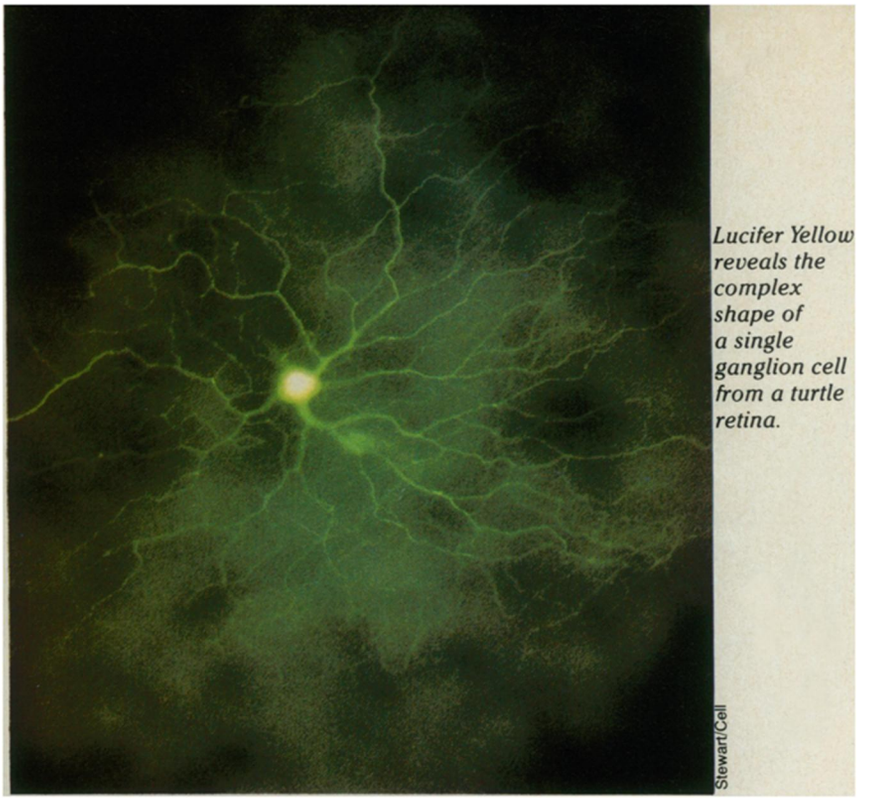


Stewart/Cell

Lucifer Yellow stays in injected mollusk cell during fixation and embedding.

Recently these same researchers have added a new dimension to the technique. In the July CELL, they report a simple manipulation of the fluorescence microscope that permits them to take pairs of slightly different pictures (by rotating a half-aperture diaphragm) of one preparation. With a stereo viewer used to view electron micrographs, each pair gives a three-dimensional display. Osborn and Weber thus have observed bundles of microfilaments crossing over and under each other and geodesic dome-like nets of microfilaments at a variety of places in the cytoplasm. In other work with Robert E. Webster, Osborn and Weber used both fluorescence microscopy and electron microscopy on the same specimen. They found that an individual fluorescent thread-like image, produced by a microtubule-binding antibody, corresponds to a single microtubule, as identified by higher resolution electron microscopy. That comparison unites electron microscopy and the fluorescence "biochemical anatomy" techniques in the task of defining different cell substituents and documenting their location within the cell.

The location and movements of organelles (subcellular structures such as mitochondria) may be directly controlled by the cell's architectural substructures. Fluorescence microscopy may be a crucial tool to unravel that relationship. Michael H. Heggeness, Melvin Simon and S. Jonathon Singer report in the August PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES strong evidence that mitochondria are associated with microtubules. Controlling the distribution of mitochondria inside a cell may be important in controlling the cell's energy supply.



Stewart/Cell

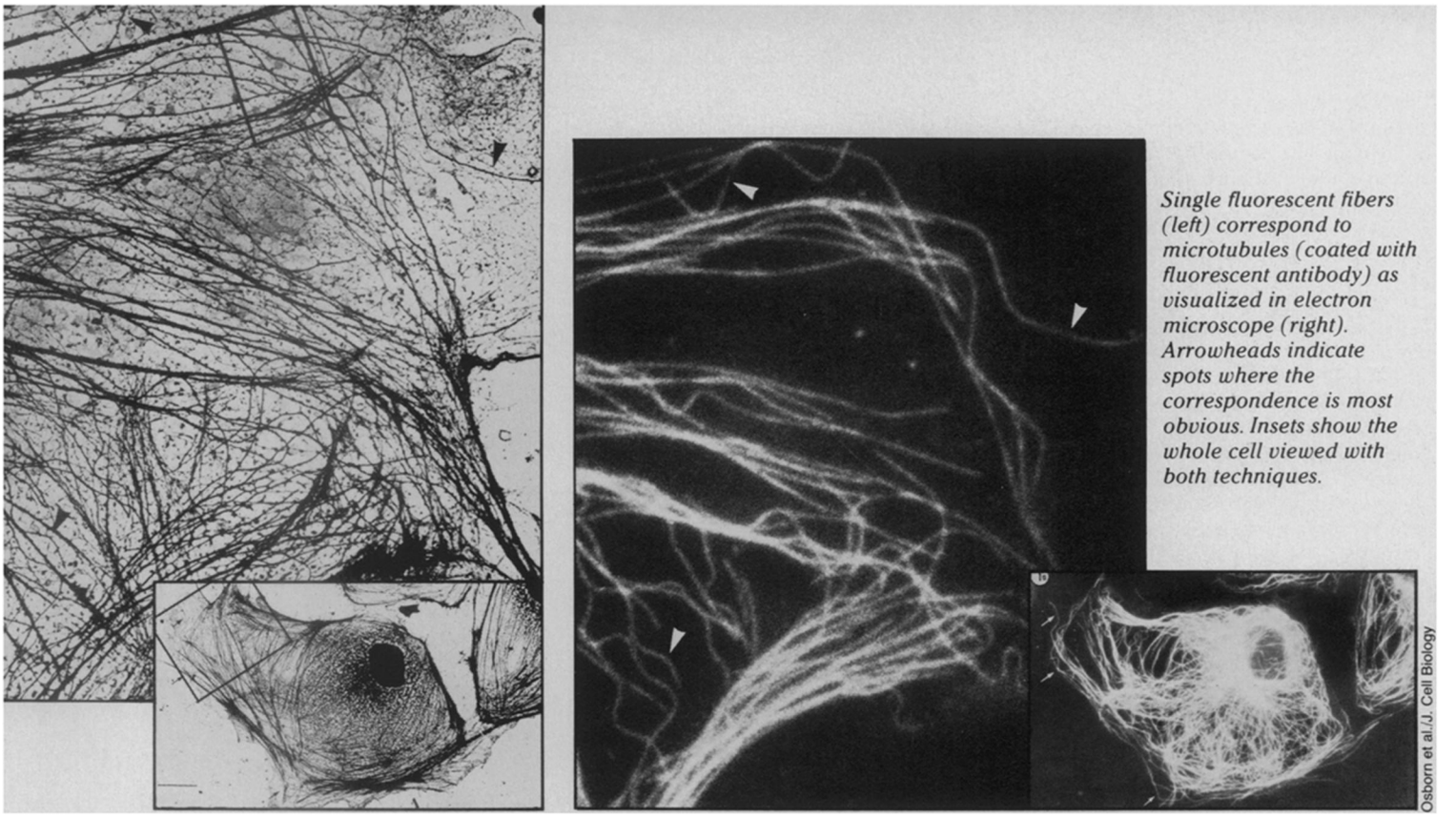
Lucifer Yellow reveals the complex shape of a single ganglion cell from a turtle retina.

In research at the University of California at San Diego, investigators labeled a variety of cells with two rabbit antibodies: antibody to microtubule protein and antibody to an enzyme characteristic of mitochondria. They next stained with rhodamine-linked goat antibody to the rabbit antibodies. The mitochondria and microtubules both fluoresced rhodamine-red, but could be distinguished by their shapes. "It was clear that in the peripheral regions of the cells the large majority, if not all, of the mitochondria were arrayed along the microtubules with their long axes generally parallel to the long axes of the microtubules," the researchers say. When they applied a drug to disaggregate the microtubules, the mitochondria retracted from the periphery to the region aound the nucleus, as if they had lost their support. That dependency is specific to microtubules; simultaneous stain with a green fluorescent dye specific for actin-containing filaments showed that the mitochondria are not aligned significantly with those fibers, Singer and colleagues found. Singer suggests that the affiliations of other organelles may be investigated through a similar approach.

Proteins on the cell surface are the target of other research by microscopists armed with fluorescent antibodies. For example, the technique has revealed that proteins in the membranes are free to move in some circumstances, but are firmly anchored in others. In experiments with spherical cells, researchers find that when any of several membrane proteins is anchored by aggregates of antibody or plant lectin, a remarkable succession of changes results. The proteins group into small patches in an energy-independent step, then consolidate into a large patch or cap in a process requiring energy. That cap is internalized by the cell.

Singer and colleage Lilly Y. W. Bourguignon have connected studies of the surface proteins and of internal structure by using two different fluorescent labels simultaneously. They mark a membrane protein with rhodamine antibody and label the internal fibers with a fluorescein-compound that binds specifically to actin. In every case they found intracellular fibers underlying first the patches of aggregated membrane protein and later the cap. Similarly, Singer, J. F. Ash and Daniel Louvard have examined membrane protein linkages in flat cells, laboratory-cultured fibroblasts. In these cases they find that the array of membrane protein molecules is not superimposed on the internal fibers until the protein molecules are clustered by antibody. "We showed that the receptors begin by being mobile. By being clustered, they are trapped and then linked across the membrane," Singer says. Thus for both spherical and flat cells Singer proposes that membrane proteins when clustered become tied to filaments beneath the surface.

Tracing chemicals in cells throughout the anatomy, or immunocytochemistry, is the third wide use of fluorescent antibody techniques. To learn which nerve cells employ a given transmitter chemical, for



Single fluorescent fibers (left) correspond to microtubules (coated with fluorescent antibody) as visualized in electron microscope (right). Arrowheads indicate spots where the correspondence is most obvious. Insets show the whole cell viewed with both techniques.

Osborn et al./J. Cell Biology

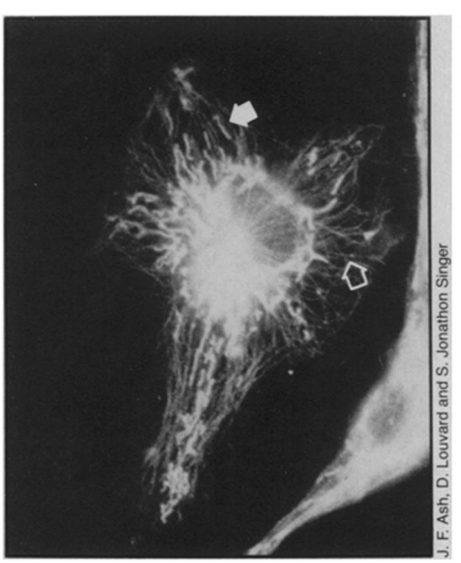
instance, the approach is to make an antibody to that chemical, stain with an indirect immunofluorescence procedure and microscopically examine the resulting distribution of fluorescence. The visualization of enkephalins, described earlier, is an example of that approach.

Analysis of neurotransmitters is one of the most exciting areas of current biology. At a recent meeting, W. Maxam Cowan, president of the Society for Neuroscience, remarked that as soon as U.S. investigators discover a new brain peptide, their Swedish colleagues (notably T. Hökfelt and collaborators) immediately publish five papers describing its distribution according to the fluorescence technique. "It will rewrite the anatomy of the brain in chemical terms," Cowan says.

The immunofluorescence techniques are limited by the specificity of antibodies used. An antibody may react with a number of compounds containing similar regions, and standard preparations may produce a number of antibodies that bind to different regions of the same molecule. Recent development of a method using laboratory-cultured cells to make a single type of antibody (monoclonal antibody production) is expected to greatly enhance the power of the fluorescence technique.

Although antibodies have extended the utility of fluorescence microscopy to a wide range of problems, simple fluorescent dyes are still *de rigeur* in some situations. For instance, physiologists poke fine electrodes into a nerve cell to measure its electrical characteristics and to eavesdrop on its signaling. But once the electrical recording is finished, researchers need to mark the cell so they can later identify it under the microscope and record its type, shape and perhaps connections.

In the July CELL, Walter W. Stewart of the National Institutes of Health reported a new dye for such cell marking. Called Lucifer Yellow, it is about 100 times more sensitive than the dyes previously available. The new dye spreads rapidly within an injected cell to light up processes far too thin to be visible with earlier methods. The brightness of Lucifer Yellow also allows complex three-dimensional structures of nerve cells to be easily viewed; pieces of tissue including the entire thickness of a cell (about 0.2 millimeters) can be examined microscopically, abolishing the requirement that the cell be thinly sliced. In addition, the dye frequently spreads from the injected cell to another, indicating a functional connection between the cells. Stewart says that Lucifer Yellow allows routine fluorescence microscopy to produce cell images that previously had only been available infrequently in the hands of the most skillful microscopists.



J. F. Ash, D. Louvard and S. Jonathon Singer

Mitochondria (solid arrows) are associated with microtubules (open arrows) in a cell fluorescently stained for both.

The demand for such a dye is so great that Stewart says his laboratory has turned into a little factory. In the last year he filled more than 100 requests for the dye from other researchers. But relief is in sight. Two chemical companies are getting ready to manufacture Lucifer Yellow, which the government has patented.

Developing the better dye took Stewart almost four years, much longer than he had expected. A satisfactory dye must be highly fluorescent, unable to cross the cell membranes and immobile during tissue fixation and dehydration. Now Stewart and colleagues plan to apply their powerful dye to some basic questions about the nervous system. They have already discovered striking patterns of cell connections in the turtle retina. Turning next to simpler animals, the researchers are attempting to create inbred strains of snails, which have large nerve cells that can be individually identified. They hope to examine the cell shapes with Lucifer Yellow in order to investigate influences of heredity and environment on the details of the nervous system.

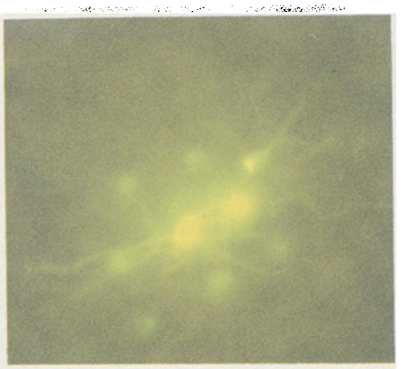
Finally, fluorescent dyes may give clues to critical membrane compositions. Rockefeller University researchers Thomas G. Easton, Jay E. Valinsky and E. Reich report in the March CELL that merocyanine 540, a fluorescent membrane probe, stains at

least 20 types of electrically excitable cells, such as nerve and muscle cells and single-celled organisms. However, with the exception of certain blood cells, it does not stain any of 13 nonexcitable cells from four species. In the electric organ of eels, only one face of each cell is excitable, and only that face is stained by merocyanine 540. "The observation that MC 540 uptake is common to excitable membranes of protozoa, invertebrates, birds and mammals makes it tempting to conclude that dye incorporation is mediated by a common fixed structural or physical property generally associated with, and perhaps essential for, the ability of membranes to propagate changes in ion flux, such as action potentials," the researchers speculate.

Thus the glow of fluorescence may not only illuminate the shapes and locations of cells and the distribution of chemicals within them, but eventually spotlight those domains within single membranes responsible for a cell's characteristic properties. □

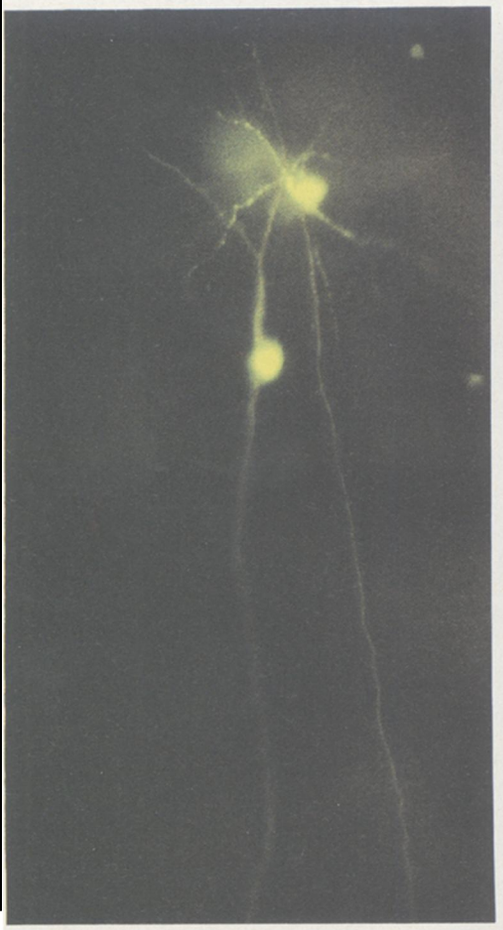
Fluroescent dye passes from injected turtle retina horizontal cell to a cluster of surrounding cells.

Easton et al./Cell

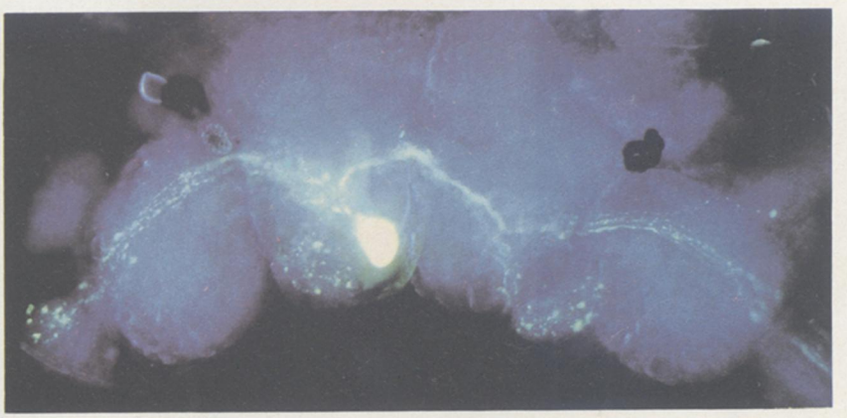


Stewart/Cell

In a honeybee eye cross-section, fluorescent dye MC 540 stains the elongated photoreceptors red-brown. But the lens and hairs reflect and scatter the incident green light so that it is visible even through the filter.



Lucifer Yellow spreads from an injected cell to another turtle retina ganglion cell.



A pattern of fine branches extends from a mollusk nerve cell heavily stained with Lucifer Yellow. A higher magnification of the dendritic branches reveals many varicosities, thought to be sites of intercellular communication.

Stewart/Cell