thionine moved eight times faster than threonine through the DFAA pool.

Loss of the C^{14}, as respired CO_2, introduces an error in these estimates. In pure-culture experiments, this loss was about double the retention in the bacteria (*9*). On 24 September 1967 a 25-ml water sample from Pamlico River was incubated in the dark for 2.0 hours with 0.100 μc of aspartic acid–C^{14}. After killing of the plankton, the water was acidified with HCl and then bubbled with CO_2. The gas evolved during incubation, plus the sparged CO_2, was passed through a $SnCl_2$ trap into an evacuated 1-liter Cary ion chamber, and the activity (microcuries) of C^{14} was measured with a Cary electrometer. About 0.004 μc was retained on the filter and 0.006 μc was respired. In uptake experiments, corrections for this error would not affect K but would lower T and increase V and v_n; thus the flux estimates (Table 1) are minimum values; the true flux may well be more than twice as high.

To refine measurements of DFAA flux, more amino acids need to be tested, measurements must be made at different seasons and depths, and accurate respiration corrections must be applied. However, comparison of our determinations of flux with published values of primary production indicates the order of magnitude of the process relative to total carbon flux through the ecosystem. Our York River DFAA data convert to a flux of 9.4 μg of carbon per liter per day; this is 1.3 percent of the surface rate of primary production reported for the same area (*10*). Corrections must be made for respiratory losses; it would then appear that we could expect DFAA flux rates to be within the range of 1 to 10 percent of primary production.

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11. Aided by the Office of Water Resources Research, U.S. Department of the Interior, and NSF grants GB-5678 and GB-6064. Contribution No. 1 from Pamlico Marine Laboratory; contribution No. 267 from Virginia Institute of Marine Science.

7 February 1968

Electron Microscopy of Unstained Biological Material: The Polytropic Montage

Abstract. *With use of an electronic picture-scanning device and a digital computer, electron micrographs taken of a specimen along several different directions can be superimposed to form a montage that is more informative than the component images. Preliminary results indicate that one may thus study unstained, unshadowed biological material at high resolution.*

Because of their low inherent contrast in the electron microscope, isolated biological particles or tissue sections are usually prepared for observation by staining, negative staining, or shadowing with some electron-dense material. While such techniques have been of great value for observation of features coarser than 20 Å, finer details are thus destroyed, obscured, or simply not enhanced. Clearly, if recent improvements in electron microscopes are to be fully utilized in biology, contrast must be enhanced without drastic molecular alteration of the specimen or obscuration by extraneous material.

One might think that sufficient contrast could be achieved by use of high-contrast emulsions and developers in making and printing electron micrographs. But the problem is not merely one of amplifying a weak "signal" representing the faint image of a specimen; there is also a form of "noise" due to fixed inhomogeneities in the specimen-support film (*1*). Photographic increase of the contrast serves only to amplify the noise along with the signal; the benefits are quite limited.

Relying upon known or anticipated symmetries in their specimen material, Markham's group (*2*) superimposed the photographic images of corresponding (symmetrically equivalent) regions within the same micrograph to make a montage in which the image, or signal, was reinforced while the background noise was largely canceled. The results are striking, but the method is applicable only to highly symmetric structures such as the simpler viruses.

I have developed another kind of montage to reinforce weak image signals which requires no specimen symmetry. The several component images that are to form the montage are obtained by tilting the specimen in the electron microscope (*3*) and taking transmission micrographs of the same area along several different directions (hence the term polytropic). To produce such a montage, the component images appearing in the original micrographs must be transformed, by simple linear operations, into images that are superimposable. If the images are reduced to numerical data with a scanning device, transformation may be by high-speed computer. Besides providing adequate contrast for the study of unstained biological material, the polytropic montage enables one to discern three-dimensional relations; a single set of component images can be combined so as to reinforce the specimen details occurring in any plane of interest.

A dilute suspension of tobacco mosaic virus and colloidal gold in distilled water was sprayed on a carbon-parlodion film and air-dried. The specimen mount supporting the film was a platinum disk (*4*) having a 70-μ viewing hole at its center. Two diametrically opposite notches cut in its edge allowed the disk to be rotated by a special wrench. The specimen holder was specially designed to hold the disk at a fixed angle of tilt, 20 deg from horizontal. By rotating the disk within its own plane, about its own axis, one changed the specimen orientation relative to the vertical electron beam. During a series of micrograph

exposures, each such rotation of the disk was accompanied by a counter-rotation of the entire holder about the axis of the electron beam; this was done to minimize variations in electron-optical distortion by minimizing the change, between exposures of the image position on the photographic plate.

With a nominal magnification of 40,000 and an 80-kv acceleration potential, a series of 12 polytropic exposures was made.

A small specimen region appearing in all 12 of the micrograph plates was selected for further study. With a photographic enlarger and suitable masks, the appropriate area from each micrograph was printed onto a single glass lantern-slide plate; thus the composite plate (Fig. 1A) contained 12 frames in a 3 by 4 array that occupied a 6-cm square. At four arbitrarily chosen points between frames, the plate was imprinted with fine X's to serve as fiducial marks.

Eight colloidal gold particles appearing in all frames of the composite plate were selected as reference features. With a microcomparator, the centers of these particles in each frame and the four fiducial marks were located on the plate relative to a common X,Y-coordinate system with accuracy better than 20 μ. The coordinates of the reference features were used as follows in the parallax computation:

Let (X_{ij}, Y_{ij}) be the location of the jth feature (where $j = 1, 2, \ldots 8$) as it appears in the ith frame (where $i = 1, 2, \ldots 12$) of the composite plate. Let x, y, z be a coordinate system fixed relative to the specimen. The location of the jth feature in the specimen is then (x_j, y_j, z_j). If one assumes that all the projections leading to production of the composite plate constitute linear transformations, the following relations hold:

$$X_{ij} = A_i x_j + B_i y_j + C_i z_j + D_i$$

and

$$Y_{ij} = E_i x_j + F_i y_j + G_i z_j + H_i \quad (1)$$

for all i and j within the defined ranges. The coefficients $A_i, B_i, \ldots H_i$ characterize the ith frame with respect to the direction along which the electron beam traversed the specimen, the electron-optical distortion and subsequent photographic distortion (as best they can be represented linearly), the final magnification, and the location and orientation of the frame on the composite plate.

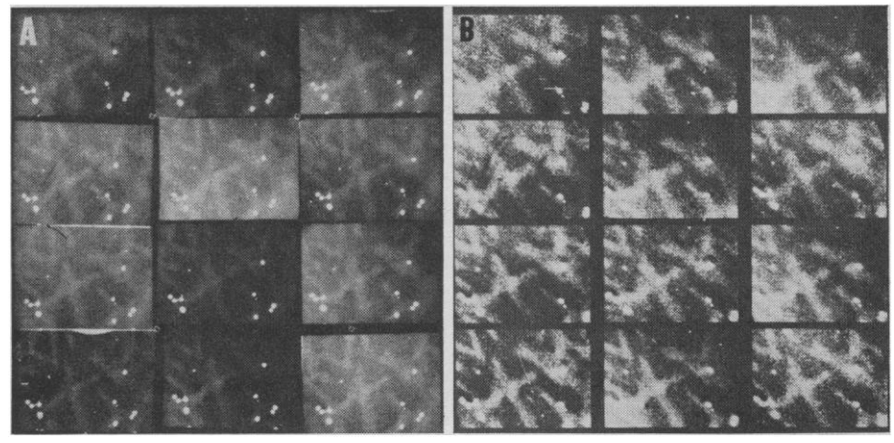

Fig. 1. (A) A print of the composite plate, showing the arrangement of its 12 frames and the four fiducial marks located between frames. The frames came from separate electron micrographs of one area of the specimen viewed in 12 different directions; thus any two of the frames may be considered a stereoscopic pair. Each frame represents a specimen field measuring about 2500 by 1900 Å. For preparation of a polytropic montage, the composite plate was scanned with a flying-spot scanner, and the readings of optical density at some 10^7 different points were recorded on magnetic tape. (B) Normalized images of the frames. After being scanned and recorded on tape, the 12 images have been adjusted so that all now have the same overall darkness and contrast, magnification, and orientation. Each frame has been stretched in an appropriate direction to compensate for its original foreshortening due to tilting of the specimen in the electron microscope.

In the parallax computation the measured coordinates of the eight gold-sol particles in the 12 frames, X_{ij} and Y_{ij}, were used for a least-squares solution of Eqs. 1. The best-fitting coefficients $A_i, B_i, \ldots H_i$ were determined for each frame, and the best-fitting coordinates x_j, y_j, z_j were determined for each feature; when the latter were substituted back into Eqs. 1, the calculated values of X_{ij} and Y_{ij} agreed closely with the measured values; the standard deviation of the error was about 8 μ.

The Eyeball, a flying-spot scanner (*5*), consists of the following components as they are arranged along the optic axis: a precision cathode-ray tube producing a 50-μ light spot on its 12.5-cm screen; a projector lens focusing an image of the light spot onto the picture to be scanned; the picture, in this case the composite plate; a condenser lens concentrating the light transmitted through the picture; and a photomultiplier that measures the intensity of the transmitted light. Between the cathode-ray tube and the projector lens there is also a semi-mirror that sends a fraction of the light along a reference leg lying normal to the main optic axis. An auxiliary photomultiplier, located on the reference leg, then receives a light signal proportional to the light incident on the composite plate. The difference between the logarithms of the light intensities received by the two photomultipliers provides a measure of the optical density of the composite plate at the point being scanned.

The Eyeball is wired to a PDP-1 digital computer (*6*) which, through appropriate instructions, specifies the XY-position of a spot on the screen of the cathode-ray tube (from among a 4096^2-array of addressable points), then causes a pulse of light to be generated at that spot, and finally receives a digitalized signal determined by the difference between readings of the two photomultipliers. A single execution of these steps—measurement of optical density at a particular point on the plate—requires 70 μsec.

Also connected with the PDP-1 computer is a 40-cm visual cathode-ray tube and a light pen; by suitable programing, the tube can be used to display an image of the plate while it is scanned by the Eyeball. With the light pen any recognizable feature of the plate can be located on the 40-cm screen, and its corresponding position on the Eyeball screen, given by the Eyeball coordinates XE, YE, can be determined. By this method, the Eyeball coordinates of the four fiducial marks were measured in a preliminary scan of the composite plate. In the PDP-1 computer the XE, YE values then were compared with the coordinates X, Y of the same four points as had been found with the microcomparator, and a least-squares computation determined the best-fitting matrix for transformation of

coordinates from the X, Y-system to the XE, YE-system. The matrix was then used to transform the previously calculated coefficients A_i, B_i, . . . H_i to the new coefficients A_i', B_i', . . . H_i' needed in the equations

$$XE_i = A_i'x + B_i'y + C_i'z + D_i'$$

and

$$YE_i = E_i'x + F_i'y + G_i'z + H_i' \quad (2)$$

These equations could then be used to compute the Eyeball location (XE_i, YE_i) of any point in the specimen (x, y, z) as it appeared in the ith frame of the composite plate.

With the composite plate remaining unmoved from its original position in the Eyeball, the main scan was then begun. The specimen coordinate z was arbitrarily held at zero, while the other two coordinates x and y were varied in regular increments to cover a 1023 by 767 array of specimen points. For each of these specimen points the Eyeball coordinates XE_i, YE_i were computed by Eqs. 2, and optical-density signals were obtained for a corresponding point in each of the 12 frames of the composite plate. The signals, numbering about 10^7 in all, were recorded on magnetic tape as 9-bit numbers arranged sequentially according to the specimen coordinates x and y and the frame number i.

The first step in reduction of these data was to read the tape and count the number of occurrences of the various optical-density readings in each of the frames; in effect a histogram showing frequency of occurrence versus optical density was obtained for each frame. Arbitrarily percentile cuts were then made in the histograms so that each scan point was assigned a "gray value" ranging from 0 to 7 according to its optical-density rank relative to the other points of the same frame. These computations were done with PDP-1, which then wrote a second magnetic tape, the "transcription tape," containing the gray values for all scan points. Thus the optical-density information per point was reduced from 9 to 3 bits, and the images of the 12 frames were equalized with respect to overall darkness and contrast (Fig. 1B). The images were then ready to be superimposed.

The first polytropic montages were made by superimposing the images photographically in the Eyeball (Fig. 2C). By substitution of an unexposed photographic plate at the position previously occupied by the composite plate, the Eyeball was converted to a camera for photographing light spots generated on the cathode-ray tube. With the PDP-1 computer the transcription tape was read, the Eyeball coordinates for each scan point were determined from

$$XE_i = k(x + x_{0i}) \quad YE_i = k(y + y_{0i}) \quad (3)$$

and the point (XE_i, YE_i) was given zero to seven light pulses according to its gray value. The constant k may be assigned any convenient value. For a montage of the ($z = 0$) plane of the specimen, the displacements x_{0i} and y_{0i} would all be set to zero, since the main scan was done over that plane. For a montage of any parallel specimen plane $z = z_1$, the required displacements are given by the formulas

$$x_{0i} = \frac{G_iB_i - C_iF_i}{E_iB_i - A_iF_i} \times z_1$$

$$y_{0i} = \frac{C_iE_i - G_iA_i}{B_iE_i - F_iA_i} \times z_1$$

where A_i, B_i, C_i, E_i, F_i, G_i are coefficients determined in the parallax computation. One should note that x_{0i} and y_{0i} are independent of x and y; that is, montages at different z-levels (Fig. 3) are obtained simply by shifting the component images relative to each other without distorting them.

As an alternative to photographic superimposition, the images can be superimposed digitally in the computer, but a memory considerably larger than that of the standard PDP-1 (4096 18-bit words) is required; a CDC-3600 (*7*), having two memory banks each containing 32,768 48-bit words, served. The transcription tape was read in several installments; the superimposition was accomplished digitally in the memory, with appropriate displacements of the component images; and a montage output tape was produced. With the Eyeball again used as a camera, this tape was translated into a photograph.

A montage, similar to those produced by photographic superimposition, could thus be obtained by setting the gray value of each montage point equal to the integer closest to the mean gray value of the corresponding scan points; this procedure amounts in effect to simple addition of the component images. But the main advantage of digital superimposition is that it is not restricted to the operation of addition; for example, some interesting pictures of tobacco mosaic virus were made

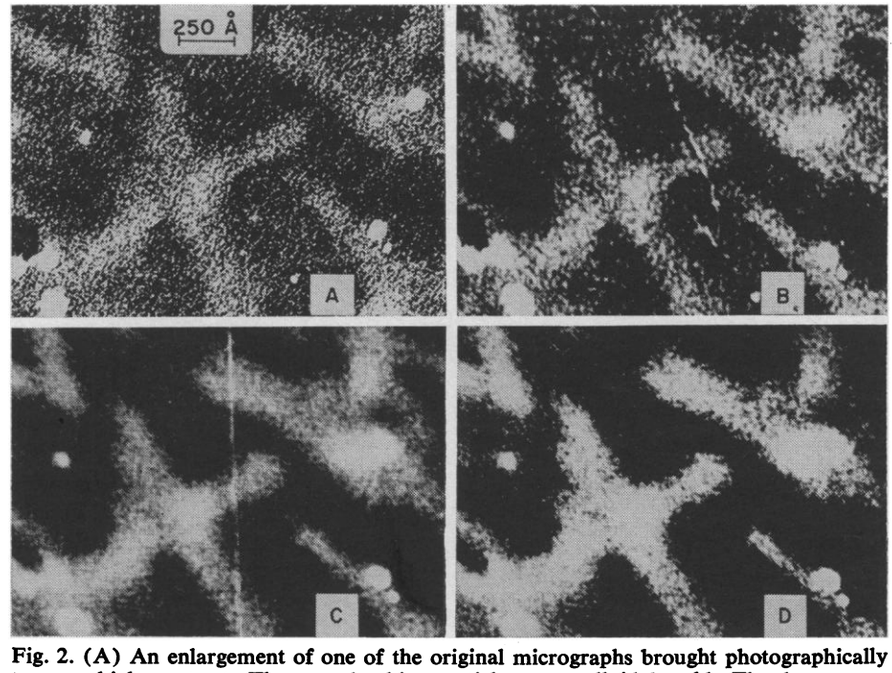

Fig. 2. (A) An enlargement of one of the original micrographs brought photographically to very high contrast. The round white particles are colloidal gold. The large gray streaks are particles of tobacco mosaic virus, unstained and unshadowed. (B) Expanded view of one of the normalized images of Fig. 1B. (C) Polytropic montage made by photographic superimposition of the 12 normalized images. (D) Polytropic montage made by correlation of the component images, point by point, in the memory banks of a computer. Note that the background noise (Fig. 2A) has been virtually eliminated, and that the virus particles are displayed with excellent contrast. The virus rods here and in Fig. 2C exhibit features similar to those seen in shadowed preparations.

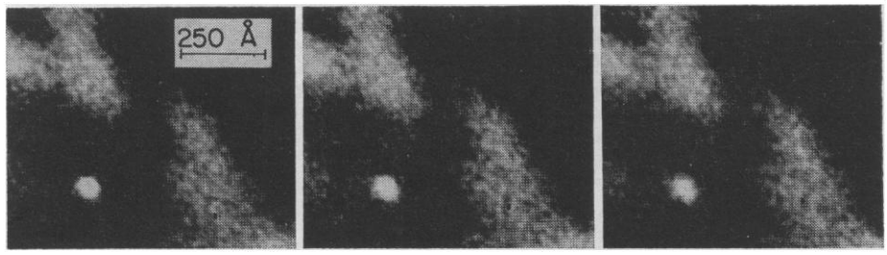

Fig. 3. Polytropic montage made by photographic superimposition of the component images in three different ways so as to accentuate the details lying at different levels in the specimen. With change in level, the gold-sol particle (bottom left) goes out of focus; virus particles, while maintaining a constant general arrangement, are changed with respect to their fine detail.

(Fig. 2D) by application of a simple correlative test to the 12 components of each montage point: let N be the number of component points with gray value higher than 5, and let the gray value of the montage point equal $(N-5)$ if $(N-5)$ is positive or let the gray value of the montage point be 0 if $(N-5)$ is negative or 0.

As a means of enhancing contrast, the usefulness of the polytropic montage is well demonstrated by Figs. 2 and 3. Good, high-contrast images have been obtained of unstained, unshadowed particles of tobacco mosaic virus. I anticipate that even smaller biological particles may be similarly imaged by use of a larger number of component micrographs taken of a specimen in various directions.

Although I have not yet objectively tested resolution for a polytropic montage, there is every reason to expect that it will be limited only by the resolution quality of the original micrographs. By comparison other factors, such as the finite size and number of scan points, nonlinearity in the cathode-ray tube of the Eyeball, and errors of image displacement, can be made negligible simply by increasing the magnification of the micrographs and scanning a correspondingly smaller area of the specimen.

Closely related to the question of resolution is that of depth discrimination. From geometrical considerations one can see that the montage has a certain "depth of focus" analogous to that of a camera. Two factors determine the degree of depth discrimination: the fineness of the details under consideration, and the angle of tilt, which is geometrically analogous to the F number. For example, if the tilt angle were increased from its present 20 deg to 45 deg, the depth discrimination, for the finest details observable, would be comparable to the lateral resolution and would be limited by only the quality and number of the original micrographs.

Thus the polytropic montage seems to offer a means of determining the three-dimensional structures of low-contrast biological specimens at a resolution of 3 Å, or the best resolution attainable with existing electron microscopes. I have not yet reached this point, but preliminary efforts have produced images of tobacco mosaic virus comparable in fineness of detail to those obtained by shadowing a similar specimen with tungsten or rhenium (*8*). Still to be determined is the extent to which the fine details appearing in the montage represent real structures of the virus rather than residual noise that may have survived this attempt at its elimination.

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References and Notes

1. Other sources of pictorial noise are photographic grain, and the ultimate inhomogeneity of the electron beam composed as it is of discrete particles. If the specimen is stable in the electron beam, both these kinds of noise can be reduced to any desired level by superimposition of a sufficient number of micrographs of the same area of the specimen.
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29 January 1968

Human Hormone Production in vitro

Abstract. *A new in vitro hormone-synthesizing cell system, line BeWO, derived from human gestational choriocarcinoma, has been permanently established; a high degree of synthesis of functional hormone has been maintained for 18 months in tissue culture. Predominantly glycolytic metabolism, dependent on the level of glucose in the medium, has been observed. Many facets of placental and tumor-cell metabolism, growth, and differentiation are found in this unique multipotential cell.*

The trophoblast of the human hemochorial placenta performs many functions essential to support of the developing embryo. Most fundamental among the absorptive, nutritive, and biosynthetic functions is the production of glycoprotein hormone. With the tissue-culture system it was demonstrated (*1*) that the pregnancy gonadotropin was synthesized in the placenta rather than in the maternal pituitary. This hormone closely resembles the luteinizing hormone of the anterior pituitary in both biological and molecular characteristics.

Establishment of continuous strains from the normal trophoblast that would provide a reliable source of hormone for clinical and research purposes has been unsuccessful. The placental cell type associated with production of human chorionic gonadatropine (HCG) has been identified (*2*), but it has not yet been possible to propagate continuously this cytotrophoblastic stem cell of the normal placenta.

Of the two villus epithelial cells, the cytotrophoblast and syncytiotrophoblast, the former represents a stem cell and gives rise to the latter by a process of differentiation (*3*). The earliest and most fundamental property of the trophoblast is synthesis of HCG. This glycoprotein is capable of maintaining the corpus luteum function of the ovary until the time of complete placental autonomy.

A rare and aggressive malignant tumor of the placenta, chorioepithelioma, is characterized by production of large quantities of the pregnancy hormone detected in patients bearing this tumor immediately postpartum. Histologically this tumor comprises many mitotically active cytotrophoblasts with moderate degrees of syncytial differentiation. No biological differences have