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Fast Axonal Transport in Extruded Axoplasm from Squid Giant Axon

Abstract. *Development of video-enhanced contrast–differential interference contrast for light microscopy has permitted study of both orthograde and retrograde fast axonal tranport of membranous organelles in the squid giant axon. This process was found to continue normally for hours after the axoplasm was extruded from the giant axon and removed from the confines of the axonal plasma membrane. It is now possible to follow the movements of the full range of membranous organelles (30-nanometer vesicles to 5000-nanometer mitochondria) in a preparation that lacks a plasma membrane or other permeability barrier. This observation demonstrates that the plasma membrane is not required for fast axonal transport and suggests that action potentials are not involved in the regulation of fast transport. Furthermore, the absence of a permeability barrier surrounding the axoplasm makes this an important model for direct biochemical, pharmacological, and physical manipulations of membranous organelle transport.*

The elongate form of the neuron has made it a valuable system for the study of intracellular transport (*1, 2*). The processes of intracellular transport may take many forms, from the movements of membranous organelles to cytoskeletal translocations, and the underlying molecular mechanisms of such movements are poorly understood. Understanding of cellular phenomena at the molecular level requires model systems that permit direct analysis of the cellular elements as well as biochemical and pharmacological manipulations, and that can be maintained long enough to conduct the manipulations. No such models have been available for the study of most types of intracellular transport.

Fast axonal transport is the movement of tubulovesicular membranous organelles from the cell body to the axonal extremities at rates of 1 to 5 μm per second (*1–3*). Many of these membranous organelles are vesicles, 30 to 50 nm in diameter, which can be identified only by electron microscopy. However, the dynamics of structures are generally lost during fixation for electron microscopy and indirect methods of analysis, such as biochemical dissection of labeled materials, have not been sufficient to provide answers about the molecular mechanisms of movement. One major difficulty stems from the presence of a plasma membrane and connective tissue sheath containing glial cells which complicate manipulation and analysis of intracellular transport processes in the axon. These structures effectively insulate the axon from external influences. Thus, to study the molecular mechanisms of fast axonal transport, the minimum requirements of a model are (i) the means to detect moving 30- to 50-nm vesicles and (ii) a preparation lacking permeability barriers.

We report here on a new preparation that satisfies both of these requirements. Recent developments in video-enhanced contrast–differential interference contrast (AVEC-DIC) microscopy permit detection of structures as small as individual microtubules, 25 nm in diameter, in living cells (*4*). It has been possible to analyze the movements of the 30- to 50-nm vesicles in cultured vertebrate neurons and intact giant axons of the squid (*5, 6*). The movements of these structures were found to correspond to fast axonal transport with respect to both rate and direction of movement, but the permeability barriers of these preparations were intact. However, it is possible to separate a cylinder of axoplasm of the squid giant axon from its plasma membrane and connective tissue sheath by mechanical extrusion (*7*). This extruded axoplasm maintains its structural integrity and many metabolic activities in vitro (*8*). The elimination of permeability barriers without the use of detergents permits precise control of experimental conditions.

The axonal transport processes in extruded axoplasm and in the intact giant axon show no qualitative differences initially. If the axoplasm is extruded directly onto a cover glass, with care being taken to maintain original dimensions and orientation, the resulting image reveals more structural detail than images of the intact axon because there is no intervening tissue to scatter and depolarize light (*5*) (Fig. 1). Otherwise, the images of extruded axoplasm are indistinguishable from the images obtained with intact axons. Electron micrographs (*8, 9*) confirm that extruded axoplasm retains the morphology of axoplasm in the intact axon.

Video records of extruded axoplasm (Fig. 1, a to d) indicate that three classes of particles can be distinguished by size and characteristics of movement [see (*5*)]. The sizes and velocities of the small, medium, and large particles are comparable in intact axons and extruded axoplasm. Prominent linear elements, which appear to delineate pathways that most particles tend to follow, can be seen more clearly in extruded axoplasm than in the intact axon. Optical sections (200- to 400-nm intervals) suggest the presence of strata that differ in their complement of membranous organelles and linear elements as well as in the predominant directions of movement.

The continuation of fast axonal transport processes in the extruded axoplasm demonstrates unequivocally that the movements of membranous organelles do not require an intact plasma membrane or a membrane potential. This is in accord with previous studies (*10*) that suggested that the rate of fast transport did not change with electrical activity or alterations in the membrane potential of the nerve fibers. Thus fast axonal transport is not regulated by the number of action potentials traversing the axon. The apparent absence of regulation of fast transport by neuronal activity is consistent with the hypothesis that it operates at or near the maximal rate. This is a difference between fast transport and other forms of motility that are regulated.

The linear elements, which are thought to be microtubules and neurofilaments, are more apparent and easily visualized in the extruded axoplasm (Fig. 1a) than in intact axons. Most re-

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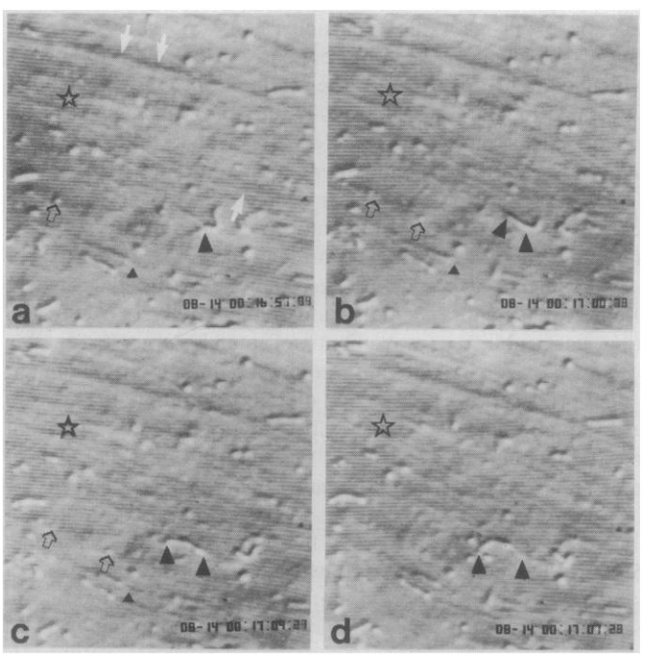
gions of the axoplasm contained particles moving in both orthograde and retrograde directions. Moreover, particles of several different sizes appeared to move along the same pathway at different times and in different directions. There were, however, variations in the proportion of particles moving in the two primary directions. Axoplasm cannot be treated as a homogeneous structure; some regions showed relative enrichment in the number of mitochondria, the amount of material moving retrograde, or some other parameter. Particle movements were not restricted to pathways defined by visible linear elements (Fig. 1, a to d), although linear elements with different orientations would not have been visible and cannot be excluded at present. Particles of all sizes could be seen to switch pathways and even to travel orthogonally across linear elements. Empirically, pathways were most rigorously defined by the movements of particles.

In intact axons and carefully extruded axoplasm, the linear elements were generally in bundles of parallel fibers, but the absence of a connective tissue sheath and plasma membrane around the axon permits direct mechanical manipulations of structures in the axoplasm. When the linear organization of extruded axoplasm was disrupted by stirring with a needle before observation, a meshwork of fibrillar elements with random orientations was produced (see Fig. 2, a to b). Particle movement continued to be active and directed for all classes, but there were no longer preferred directions. Most of the particles moved in close apposition to fibrillar structures, but particles switching between fibers and moving in regions with no visible linear elements could also be observed.

If extruded axoplasm is protected from drying out, particle movement continues for several hours. If axoplasm is perfused repeatedly with appropriate buffers, particle movement is maintained for an hour or more. The buffers used in perfusions preserve the structural integrity of extruded axoplasm for at least 24 hours (*8*). Particle movements eventually cease after prolonged extractions (significantly more than 1 hour) in excess volumes of buffer (five or more volumes). This may be due to extraction of polypeptides in the absence of permeability barriers (*8*). The longevity of the extruded axoplasm preparation is in contrast to the shorter useful life spans of models made permeable with detergents, for example, that can be used for study of intracellular transport (*11*) but may last only minutes.

In extruded axoplasm, movements may be evaluated in a defined environment and ionic conditions may be altered or pharmacological agents introduced several times in a single preparation. For example, perfusion with the oxidative phosphorylation uncoupling agent, 2,6-dinitrophenol (DNP; 200 μM), rapidly inhibits the movement and induces swelling of the large particles in both the presence and absence of added adenosine triphosphate (ATP) (1 mM). The inhibition is reversed by removal of DNP and addition of ATP or adenosine diphosphate (1 mM). By contrast, movement of the small- and medium-sized particles is unaffected as long as ATP is present. The requirement of ATP for movement is in accord with earlier studies (*10*). The effects of DNP confirm the identification of large particles as mitochondria and raise the possibility that the movement of mitochondria is coupled in some way to the continued synthesis of



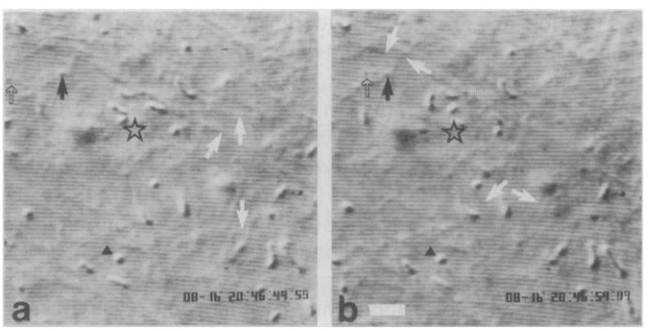


Fig. 1 (left). (a to d) Photographs taken from the video record illustrating particle movement in extruded axoplasm. The field is approximately 20 μm and the numbers indicate the date (month and day) and time elapsed (hours, minutes, seconds, and hundredths). All video was recorded in real time. This axoplasm was extruded 25 to 30 minutes before the records were made. In the original axon, the cell bodies would have been toward the lower right, so that orthograde transport would be moving from right to left along the diagonals created by the bundles of linear elements (white arrows) corresponding to microtubules and neurofilaments. The large triangles mark the movement of a large particle (mitochrondrion) with the original position of the leading edge designated in (a) and the same position marked by the right-hand triangle in (b) to (d). This large particle moves both in the orthograde and lateral directions, crossing several sets of linear elements in the process. The small triangle marks a mitochondrion that undergoes an elastic recoil event as described in (*5*). The open arrow marks the movement of a medium-sized particle in the retrograde direction with the left arrow in (b) and (c) indicating the original position. The movements of the small particles are difficult to follow in still photos because of their low contrast and small size. The area above the star represents a relatively active area, and changes in the distribution of small, low-contrast figures can be seen even in photographs. In the video record, these small particles can be seen to move throughout the field. Fig. 2 (right). (a and b) Photographs from the video record of extruded axoplasm that was mechanically disrupted prior to observation. The field and viewing conditions are similar to those in Fig. 1, but the axoplasm was disrupted with a needle prior to observation. The linear elements (white arrows) are no longer arranged in parallel bundles of fibrillar structures (see Fig. 1a); instead they form a meshwork of randomly oriented linear elements. Movements continue to occur for all classes of particles, although net movements tend to be shorter and no preferred directions are apparent. The large particles (mitochondria) in the area above the star rearrange considerably. The filled and open arrows indicate the positions of two medium-sized particles at this 10-second interval. The region above the small triangle contained a large number of small particle movements in this 10-second interval. See text for further discussion.

ATP by the mitochondria. It is not known whether this results from the loss of some chemical coupling or from the swelling of the mitochondria that prevents passage through the matrix of the axoplasm.

The use of AVEC-DIC microscopy permits direct analysis of all the moving, membranous organelles for the first time. To our knowledge, no other microscopy system is available that can detect the full range of membranous organelles in living preparations. Removal of the plasma membrane and other permeability barriers by mechanical extrusion makes biochemical, pharmacological, and mechanical manipulations possible without the extraction of components with detergents. The resulting preservation of polypeptide composition and organization in the native state results in a greatly extended life span for the model. Therefore, this model fulfills all the criteria for a study of the molecular mechanisms of intracellular transport of membranous organelles.

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In vivo Identification of the Transforming Gene Product of Simian Sarcoma Virus

Abstract. *Simian sarcoma virus (SSV) deletion mutants were constructed from a molecular clone containing the entire infectious provirus. Transfection analysis of these mutants localized the SSV transforming gene to a small region of the viral genome encompassing its cell-derived sequence (v-*sis*). Antiserum to a peptide synthesized on the basis of the predicted amino acid sequence of the SSV transforming gene detected a 28,000-dalton protein that was specifically expressed in SSV transformed cells and that corresponded in size to that predicted from the v-*sis *coding sequence. The v-*sis *gene product designated p28^{sis} was not a phosphoprotein, nor did it possess detectable protein kinase activity. These findings distinguish p28^{sis} from a number of other retroviral* onc *proteins.*

Acute transforming retroviruses have been isolated from a number of vertebrate species. These viruses cause sarcomas or hematopoietic tumors, but in some cases induce carcinomas as well (*1*). Simian sarcoma virus (SSV) is the only known acute transforming retrovirus of primate origin (*2*). The isolation of biologically active molecular clones containing the intact, integrated SSV genome has made it possible to approach an understanding of its transforming mechanism. Studies to date have shown that the 5.1–kilobase pair (kbp) SSV genome arose by recombination of simian sarcoma associated virus (SSAV) with a 1-kbp segment (v-*sis*) derived from a woolly monkey cellular gene (*3–5*). By analogy with information available concerning the cell derived sequences of other transforming retroviruses, v-*sis* might be expected to play an important role in transformation induced by SSV. Nucleotide sequence analysis of v-*sis* has revealed a long open reading frame that could code for the SSV transforming protein (*6*). Our studies were undertaken to establish directly the role of v-*sis* in SSV induced transformation and to use knowledge of the primary nucleotide sequence of v-*sis* to identify the SSV transforming protein.

To localize the region of SSV required for transformation, we constructed various deletion mutants from a molecular clone of SSV DNA and tested their ability to transform NIH/3T3 cells in a transfection assay (*7*, *8*). The intact viral genome (pSSV-11) exhibited a transforming activity of $10^{4.1}$ focus-forming units (FFU) per picomole of viral DNA (Fig. 1). A subgenomic clone, pSSV 3/1, from which the 3′ long terminal repeat (LTR) was deleted, showed no reduction

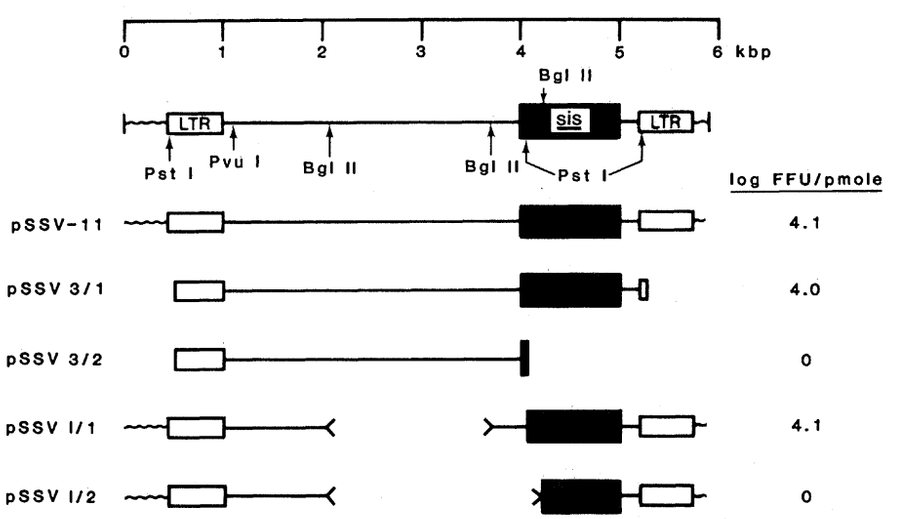

Fig. 1. Construction and biological analysis of SSV deletion mutants. The integrated form of SSV was excised from λ-SSV-11 Cl 1 (*3*) and purified by elution from a preparative agarose gel. Deletion mutants pSSV 3/1 and pSSV 3/2 were constructed by cloning products of a reaction in which purified SSV DNA was partially digested with Pst I. The intact viral genome, pSSV-11, was obtained by cloning the λ-SSV-11 Cl 1 insert at the Eco RI site of pBR322 (*30*). pSSV I/1 and pSSV I/2 were constructed by limited Bgl II digestion of pSSV-11 followed by religation. In each case, the structure of individual deletion mutants was determined by restriction enzyme and Southern blotting analysis. Transfection of NIH/3T3 cells with plasmids containing SSV wild-type or mutant DNA's was performed by the calcium phosphate precipitation technique (*7*) as modified by Wigler *et al.* (*8*). Transformed foci were scored at 14 to 21 days.

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