RETROSPECTIVE

Watt W. Webb: His measurements of the seemingly inaccessible broadened the horizons of biophysics

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Watt W. Webb's scientific career was a series of triumphs over challenges to make difficult measurements of important physical and biological phenomena: "impossible problems of experimental physiology," as he described them (1). A common theme was his use of light microscopy in new ways to reveal equilibrium and dynamic properties in biomolecular systems and in organisms. Webb's deep understanding of physics directly contributed to new instruments and methods for detecting and interpreting the hitherto undetectable and undecipherable. Webb and his collaborators opened approaches adopted far and wide to investigate important questions in areas of physics, biology, biochemistry, and biophysics.

Webb was born on August 27, 1927 in Kansas City, Missouri. He entered the Massachusetts Institute of Technology at 16, where he majored in business and engineering administration. He then worked as an industrial engineer at Union Carbide. During that period, he resumed studies at the Massachusetts Institute of Technology and by 1955 had completed a Doctorate of Science in materials science physics and mathematics.

Early Engineering Physics

Beginning in the late 1950s and continuing through the early 1970s, Webb contributed to a wide variety of physics topics, including crystal growth and dislocations, magnetization, continuous transitions at critical liquid–vapor interfaces, and fluctuations in superconductors. Even at this early stage in his career, Webb showed an interest in statistical fluctuations, albeit in quantum systems, statistical noise, and phase transformations that prefigured his later work on more biologically oriented areas.

Entering Biophysics—Fluorescence Correlation Spectroscopy

Webb's entry into biophysics in the early 1970s was driven by a tantalizing challenge: How to measure the kinetics of chemical reactions without displacing them from equilibrium. Even in equilibrium the concentrations of chemical reactants fluctuate spontaneously about their equilibrium values. The reaction kinetics could be determined from the time courses of these tiny fluctuations. Webb's interest in the problem may have reflected his earlier work on fluctuations in quantum systems (2). These later studies were originally motivated by questions about the kinetic mechanism of the helix to random coil transition of DNA

Watt W. Webb. Image credit: Cornell University, licensed under CC BY-NC-ND.

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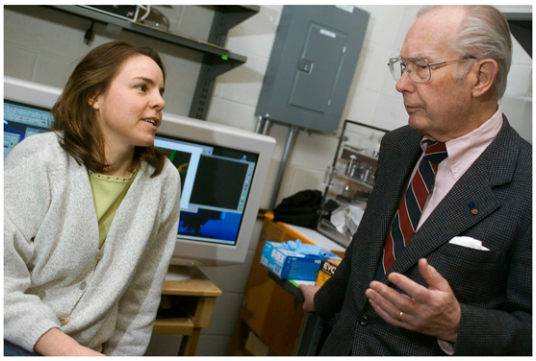

Watt W. Webb in his laboratory. Image credit: Cornell University, licensed under CC BY-NC-ND.

molecules (3). A promising simpler reaction system with which to begin, however, was the binding of ethidium to DNA, which causes a large enhancement of the ethidium fluorescence (4). Work on this system led Webb and coworkers to develop fluorescence correlation spectroscopy (FCS) (5–7). FCS at that time was difficult and was never suitable to measure fluctuations of DNA helicity. Nevertheless, FCS is now routine in many laboratories throughout the world and is being applied to a wide range of subjects (8).

Although the main motivation for developing FCS was to measure chemical reaction kinetics via fluctuations, an easier application, which is still of great interest, is to measure translational diffusion via the fluctuations of fluorescence that arise as fluorescent molecules diffuse across the laser beam that measures the sample fluorescence. The small size of the laser-illuminated spot makes FCS particularly suited to measuring diffusion in small systems (e.g., within or on the surfaces of biological cells). One of most elegant aspects of FCS is its extraction of macroscopic rate parameters from microscopic fluctuations. Related work on fluorescence fluctuations due to molecular rotational motion was also proceeding at this time (9). It is sometimes easier, however, to measure the response to displacing the system from equilibrium macroscopically by photobleaching a faction of the fluorophore within the focal spot and then measuring the reequilibration of the fluorescence due to diffusion back into the bleached region. This is the basis of fluorescence photobleaching recovery, now called fluorescence recovery after photobleaching (FRAP) (10, 11). This approach was also developed with technical variations independently around this time by other laboratories (12–14).

This work demonstrates Webb's strength in the design of measurement approaches and his experience in the interpretation of microscopic properties of physical systems. These were crucial to the development of FCS and were hallmarks of his many and important later contributions to molecular and cellular biophysics.

Diffusion of Cell Membrane Components and Membrane Lipid Phase Behavior

The availability of FCS and FRAP allowed Webb and his coworkers to address a major set of subjects still currently of interest to biophysicists and biochemists: Measurement of the diffusion rates and molecular interactions of lipids and proteins on and within cell membranes. The fluid mosaic model of membrane proteins embedded in a fluid lipid bilayer (15) and theory (16) suggested that the diffusion rates of the proteins should be comparable to that of the membrane lipids. FRAP and FCS measurements found that protein diffusion was much slower than expected (17, 18). Since then, many studies using FRAP, single-particle tracking, and other methods have been focused on this subject.

Hearkening back to his early work on phase behavior of nonbiological fluid mixtures, Webb and his colleagues studied the phase equilibria of lipid bilayer membranes (19–21). Lipid molecules with diverse polar head groups, and both saturated and unsaturated hydrocarbon chains of varying lengths, yielded membranes with subtle differences in phase behavior. Experiments were carried out on giant unilamellar vesicles, single lipid bilayer "bubbles" 10 μm or more in diameter. Fluorescent lipid molecules that partitioned selectively into (quasi-solid) gel and fluid phase domains were essential for this work (20, 21). These studies revealed informative relationships between the shapes of domains, their lipid compositions, the interfacial tension of domain boundaries, and membrane curvature. Moreover, they demonstrated that the compositions of the two phases could approach one another until, at a critical temperature, the distinguishable phases disappear in a continuous phase transition. Measurement of thermal shape fluctuations of the giant unilamellar vesicles led to evaluation of their curvature elastic modulus (22).

Multiphoton Microscopy

Confocal fluorescence microscopy makes possible the detection of a small number of molecules and therefore was essential to the development of FCS and FRAP (11). Moreover, laser-scanning confocal microscopy provided an important and widely used tool for visualizing cells beginning in the 1980s. The Webb laboratory pioneered the next major advance in fluorescence microscopy, multiphoton microscopy (MPM) (23–26). MPM provided new capabilities both for scientific investigation and for diagnostic and surgical uses. Returning to engineering practical contributions, Webb devoted considerable effort to develop endoscopic uses of MPM. A Google Scholar search on "Watt Webb, endoscopy" returns many papers devoted to endoscopic instrumentation (27) and to the evaluation of diagnostic studies, such as the areas of human bladder cancer (28) and lung cancer (29).

MPM consists of combining two or more photons absorbed essentially simultaneously to excite fluorescence. The sum of the energies of the absorbed photons must match the total energy required for one-photon excitation of the fluorescence. Hence, the wavelength of each of the combined photons is greater than the wavelength required for conventional one-photon excitation. Mode-locked lasers are needed to produce short but very intense light pulses needed for the very high photon fluxes required for

the simultaneous absorption of two or more photons. Substantial advantages accrue from the use of a longer wavelength of exciting light. The background fluorescence excited by the light of longer wavelength as it travels through the medium toward and from the focal point is much less than would arise from one-photon excitation at a shorter wavelength. In addition, photobleaching of the fluorophore would be confined to the focal volume rather than over the entire path of the excitation light. Furthermore, because excitation occurs only in the focal volume, even scattered fluorescent light can be used to construct the image. As a result, a major advantage of multiphoton technology is its suitability for deep tissue imaging (30). Webb's long and diverse experience with lasers was especially beneficial to the development of MPM.

Another version of MPM uses second harmonic generation (SHG) in which two photons of the same frequency of light are combined by a nonlinear material to generate a photon of twice the frequency (energy). Early applications of this phenomenon were carried out by Webb and his coworkers, including studies of collagen fibrils, which generate a strong SHG signal (31) and are useful to analyze how cells remodel collagen matrices in engineered tissue constructs (see, e.g., ref. 32). SHG is also useful to measure fast action potentials (33) and uniformly polarized microtubules in brain tissue (34).

Electrophysiology

Even a brief account of Webb's contributions should not omit his work on electrophysiology, including an early use of patch clamping to record conductance by chloride channels from electric eels in reconstituted lipid vesicles (35). Related work included studies of the "giga-seal" high-resistance adherence of the channel-containing membrane to a micropipette, essential for these measurements (36), and also the effect of membrane tension on conductance by alamethicin channels (37). More directly, biological studies addressed auditory mechanisms in crickets (38, 39) and in the saccular hair cells of the frog inner ear (18, 26, 40–42).

Concluding Thoughts

Watt W. Webb made many important and diverse contributions to biophysics, including the development of methods for measuring difficult phenomena, such as the kinetics of spontaneous concentration fluctuations in chemical reaction systems, MPM, membrane phase equilibria, and auditory transduction mechanisms, and the interpretation of these measurements to provide mechanistic understanding of important biochemical and biophysical phenomena. His work provides a roadmap to some of the most interesting and significant work in these areas to occur over the last half century. This accounting would be incomplete, however, if I failed to mention Webb's great personal influence as a teacher and mentor to a large cadre of contemporary biophysical scientists. To his students and collaborating scientists, Webb was always a supportive friend and source of sound guidance. Those of us who had the pleasure of visiting or working in his laboratory in the basement of Clark Hall at Cornell University will always remember and treasure the pleasure of seeing a difficult measurement yield a long-sought result and the lively discussions of science and many other topics during the 4 o'clock coffee hour.

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