A NEW MINIATURE HYDROSTATIC PRESSURE CHAMBER FOR MICROSCOPY

Strain-Free Optical Glass Windows Facilitate Phase-Contrast and Polarized-Light Microscopy of Living Cells.

Optional Fixture Permits Simultaneous Control of Pressure and Temperature

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ABSTRACT

This paper describes the development of a miniature, temperature-controlled, stainless steel pressure chamber which uses strain-free optical glass for windows. It is directly adaptable to standard phase-contrast and polarized-light microscopes and requires a minimum amount of equipment to generate and measure pressure. Birefringence retardations (BR) of 0.1 nm up to 3,000 psi, 0.4 nm up to 5,000 psi and 1.0 nm up to 10,000 psi can be detected over a 0.75-mm central field with two strain-free Leitz 20x UM objectives, one used as a condenser. In phase-contrast studies a Nikon DML 40x phase objective and Zeiss model IS long working-distance phase condenser were used, with little deterioration of image quality or contrast at pressures as high as 12,000 psi. The actual design process required a synthesis of various criteria which may be categorized under four main areas of consideration: (a) specimen physiology; (b) constraints imposed by available optical equipment and standard microscope systems; (c) mechanical strength and methods for generating pressure; and (d) optical requirements of the chamber windows. Procedures for using the chamber, as well as methods for shifting and controlling the temperature within the chamber, are included.

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been confined to low resolution bright-field microscopy.

Marsland (1950, 1970) and Kitching (1954) have designed optical pressure chambers and have used them successfully with bright-field microscopy to study changes in cell shape and the inhibition of cell cleavage by pressure (Marsland, 1970; Kitching, 1970). Both investigators studied changes in relatively large dimensions and did not employ special techniques for enhancing image contrast. Because their chambers are large, especially the Marsland chamber, they cannot be used directly with standard microscopes, and special microscope systems had to be constructed. Kitching's chamber, with its smaller size and close apposition of the windows, allowed optics with higher numerical aperture to be used, and thus, gave better resolution than was possible with Marsland's chamber. Unfortunately, the window glass in Kitching's chamber developed internal cracks that produced image aberrations and reduced resolution. Also, the optical anisotropy produced by the internal cracks and high lateral stress caused by the conical window support would interfere seriously with the contrast-producing mechanisms of either phase-contrast or polarized-light microscopy.

Information about pressure-induced morphological changes in small organisms such as bacteria, or changes in internal cell structure, has frequently been obtained by observing specimens that were fixed or stabilized immediately after decompression. These methods of specimen preparation are inadequate since (a) most changes effected by moderate pressure (≤10,000 psi) are rapidly reversed after decompression (Marsland, 1970; Salmon, 1975 a), (b) fixation or stabilization may induce alterations in the specimen, and (c) dynamic aspects of cell processes are difficult to assess.

To avoid these problems, a new microscope pressure chamber was designed and constructed for the specific purpose of observing directly the effects of pressure on the labile microtubules of the mitotic spindle and on the movement of chromosomes in marine oocytes (Salmon, 1975 a–c). These studies required greater resolution than was possible with the Marsland chambers and required phase-contrast optics (for observing chromosomes) and sensitive polarized-light optics (for observing and measuring birefringence retardation [BR] of the spindle) for which neither the Marsland nor the Kitching design was suitable. While the following discussion refers repeatedly to the suitability of the new chamber for the specific problems that instigated its development, the potential applications of the chamber are certainly more numerous.

The principal objectives governing the design of the chamber were: (a) to observe living cells, (b) with standard microscope systems, (c) under pressures as high as 10,000 psi, (d) with sufficient image quality to see chromosomes clearly with phase-contrast and to measure spindle BR with polarized-light microscopy. The actual design process could not be a sequential step-by-step procedure, but required a synthesis of various criteria which may be categorized under four main areas of consideration: (a) specimen physiology; (b) constraints imposed by available optical equipment and standard microscope systems; (c) mechanical strength and methods for generating pressure; and (d) optical requirements of the chamber windows. Comments on temperature changes and control, as well as procedures for using the chamber, are also included.

For reference during the following discussion, detailed drawings of the chamber are given in Fig. 1. Photographs of the constructed chamber alone and installed on a Leitz Ortholux microscope are presented in Figs. 2 and 3.

CRITERIA AND DESIGN BASED ON CONSIDERATIONS OF SPECIMEN PHYSIOLOGY

To maintain healthy living cells in a sealed chamber, the internal volume must be large enough to sustain normal cellular respiration and the chamber materials must not adversely affect cell growth and survival. The internal volume of the chamber is about 0.075 ml which is more than sufficient to meet the respiratory requirements of several hundred cells. In a variety of marine oocytes, for example, oxygen consumption varies between 75 and 350 pl oxygen per egg per hour, depending on cell type but not on cell volume (Krahl, 1950; Scholander et al., 1952). Cell development is impeded when the oxygen tension falls to 20% of normal (Amberson, 1928). At room temperature (20°C) and atmospheric pressure, water contains about 32 μl of dissolved oxygen per ml. Assuming that the cell medium fills the internal volume, the number of developing marine oocytes the chamber...
can support during a 10-h experiment is about:

$$\frac{(0.075 \text{ ml})(0.8)(32 \mu l O_2/ml)}{(350 \text{ pl } O_2/\text{egg h})(10 \text{ h})} = 550 \text{ oocytes.} \quad (1)$$

With approximately this number of marine oocytes in the sealed chamber, even after 24 h about half had survived and developed to hatching.

The use of inert fluorocarbon oils (3M Company, St. Paul, Minn.) for the specimen medium can extend the limits on number of cells or experimental duration apparently because they have a greater oxygen solubility. With Kel-F10 oil as the cell medium, *Nephrotheta suturalis* spermatoocytes lived more than 48 h in the sealed chamber, finally being overcome by the growth of bacteria.

The only materials in contact with the cell medium when the chamber is sealed are 303 stainless steel, glass, silicone cement, neoprene, or Buna-N (O-ring), and Kel-F3 fluorocarbon oil in the pump line. With proper care the 303 stainless steel did not corrode even when seawater was used as the cell medium. The chamber materials have had no apparent toxic effects on various cells including marine oocytes, insect spermatoocytes, plant endosperm cells, melanophores, and protozoa.

**CRITERIA AND DESIGN BASED ON CONSIDERATIONS OF STANDARD MICROSCOPE SYSTEMS AND AVAILABLE OPTICAL EQUIPMENT**

As mentioned previously, the chamber was designed to be used with commercially available microscope stands and optics for phase-contrast and polarized-light, as well as bright-field microscopy. Physically, the optical components in all three microscope systems are arranged similarly and can be radially symmetrical. A cylindrical chamber, which can be rotated $\pm 45^\circ$ for polarized light studies, can be used with any of them. The major design problem was to satisfy, in one chamber, the optical requirements of the specific objectives and condensers needed for each system. The principal lens characteristics important to the

**FIGURE 1** Detailed drawings of the microscope pressure chamber. The material is 303 stainless steel. All dimensions are $\pm 0.001$ in unless otherwise noted. All sharp corners broken. All surfaces machine finished smoothly with no burrs. The window seat surfaces should be turned flat within 0.0002 in.
Figure 2  The pressure chamber disassembled, showing the bayonet end-closure. Window on lower unit (left) is designed for easy specimen mounting and cleaning. The outside diameter of the chamber is only 1.25 in.

Figure 3  Pressure chamber in its temperature control stage. The chamber and stage are mounted on the Leitz Ortholux microscope used in the optical experiments described in the text. Standard stage spring clips supply more than adequate force to hold the chamber firmly against the microscope stage.

design of a chamber are numerical aperture (NA) and working distance (WD) as illustrated in Fig. 4. The numerical apertures of the desired lenses determine the necessary apertures of the window ports, and the required working distances of available lenses restrict the thickness of the chamber windows and end-restraints.

Resolving power of a microscope condenser and objective lens system is given by:

\[ d_{\text{min}} = \frac{1.22 \lambda}{NA_{\text{obj}} + NA_{\text{cond}}} \]  

where \( d_{\text{min}} \) is the minimum separating distance at which two points can be detected as distinct entities, \( \lambda \) is the wavelength of illuminating light, and \( NA_{\text{obj}} = n_i \sin \theta_i \) (\( n_i \) being the refractive index between the specimen and the lens, \( i \), and \( \theta \) being the maximum angle of acceptance of the lens as pictured in Fig. 4) (Setlow and Pollard, 1962, p. 286; Longhurst, 1967, p. 315). The greater the desired resolution, the greater must be the port aperture of the chamber window. However, since the contribution of the condenser to total NA cannot be greater than the contribution of the objective, the chamber design favored factors such as port aperture and end-restraint thickness that provided maximum objective NA, compromising when necessary on factors limiting condenser NA.

Filling the port regions with immersion oil (refractive index \( n = 1.515 \)) increases the effective port NA. Without immersion oil the objective port NA is 0.7 and the condenser port NA is 0.37. When the port regions are filled with immersion oil and sealed with a cover slip, the effective objective port NA is 0.9 and the condenser port NA, 0.56. In order to contain immersion oil in the condenser port, the outer rim of the port was undercut to hold a standard 18-mm circular cover slip. Surface tension was sufficient to hold the cover slip and oil in place.

Lenses with high numerical apertures generally require short distances between the lens and specimen. The working distance usually specified for a lens is the distance from the front element of the lens to the upper surface of a cover slip that is assumed to be 0.17 mm thick. Since the chamber windows and end-restraints are, by necessity, much thicker than a normal cover slip, the working distances of standard high aperture lenses (WD < 1 mm) are much too short. When the specimen is mounted against the objective window, the minimum objective working distance permitted by the chamber is 2.8 mm, and the minimum condenser working distance is 13.4 mm. With immersion oil, however, lenses with shorter working distances may be used: 2.3 mm for the objective and 9.5 mm for the condenser.

The thick flat windows also introduce spherical aberration. The degree of spherical aberration increases as a function of both window thickness and numerical aperture. Standard lenses are corrected for spherical aberration when a 0.17-mm thick cover slip is used. Spherical aberration is not
much of a problem with low aperture lenses, but high aperture lenses, if not available corrected for the thickness of the window, should be corrected by one of several methods (Longhurst, 1967, p. 372; Hartley, 1964, p. 85; Slayter, 1970, p. 193).

Objectives for polarized light must be strain-free, and if possible, rectified (Inoué and Hyde, 1957). Sufficient resolution and magnification for observing mitotic spindle BR in the experiments reported in Salmon 1975 a–c were obtained by using two strain-free Leitz UM 20× objectives (NA 0.33, WD 14 mm), one as a condenser. However, any strain-free 10× objective (NA 0.25, WD 6.5 mm) can be substituted for the objective lens. The working distances of these lenses are long enough so that immersion oil is not needed in either chamber port, and the numerical aperture is low enough so that spherical aberration does not require correction.

Good resolution and magnification for phase-contrast studies of chromosomes were obtained with the Nikon 40× DML phase objective (NA 0.6, WD 2.0 mm for a 1.5-mm cover glass) and a Zeiss IS phase condenser (NA 0.7, WD 11 mm). The Nikon objective comes with an adjustable collar for correcting spherical aberration for cover

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**Figure 4** Schematic diagram of microscope optical components used with idealized pressure chamber. Microscope is focused on an object in the center of the chamber. The objective NA is drawn equal to the condenser NA with the working distance of the condenser greater than the objective.
glasses from 1.3 to 1.8 mm thick. No immersion oil is needed in the objective port, but the working distance of the Zeiss IS condenser is slightly shorter than the actual thickness of the chamber window plus end-restraint so that immersion oil is necessary in the condenser port. Alternatively, a simple negative lens of -34 mm focal length (Edmund Scientific Co., Barrington, N. J.) placed underneath the condenser will also give proper Köhler illumination.

CRITERIA AND DESIGN BASED ON CONSIDERATIONS OF MECHANICAL STRENGTH AND METHOD OF PRESSURE GENERATION

We anticipated that 10,000 psi would be the highest pressure necessary for studying effects of pressure on the mitotic apparatus, so the chamber was designed to contain a maximum internal pressure of 15,000 psi as a safety margin. The ability of a cylindrical chamber to contain such pressure depends on the material yield strengths, window and wall thickness, end-closure, and sealing.

The strain-free glass optical flats (5 mm diameter, 1.75 mm thick, obtained from the Edmund Scientific Co.) were chosen primarily on the basis of optical considerations. However, with the proper size, geometrical shape, window support, and polished surfaces, glass of these dimensions is more than strong enough to contain the maximum desired pressure (Hamann, 1957; Edgerton and Hoadley, 1961; Pugh et al., 1963). There are two basic designs for windows used in pressure chambers: cylindrical windows and conical windows. Both types use the Bridgman principle of unsupported area to produce positive sealing (Bridgman, 1970). In both cases, the normal pressure in the seat area is greater than the chamber pressure by:

\[ P_s = P_c \frac{r_w^2}{r_w^2 - r_p^2}, \]

where \( P_c \) is the chamber pressure, \( r_w \) is the radius of the window, and \( r_p \) the radius of the port. Considering the defects of the conical window design discussed earlier in reference to Kitching's chamber, we chose to use cylindrical windows.

The outside diameter of the 1-mm wide window seat area is approximately 0.006 in larger than the window diameter. The windows are sealed with silicone cement, with care being taken to exclude all trapped air. This method of installation prevents the induction of stresses from thermal expansion of the chamber walls and the cement transmits the chamber pressure uniformly around the windows. The normal tensile strength of glass is less than 10,000 psi, but the compressive strength is about 100,000 psi. The high tensile stresses induced by the window bending into the port region are significantly lowered by the circumferential chamber pressure. The bursting pressure of a cylindrical glass window decreases as the ratio of thickness to port diameter decreases. The window port diameter as designed is 3 mm, giving a ratio of 0.6. The bursting pressure has been determined for similar window designs to be about 40,000 psi (Pugh et al., 1963) which is considerably higher than the chamber pressures anticipated. The chamber windows have proved more than adequately strong. After about 1,000 pressurizations, some as high as 15,000 psi, there has been no breakage, cracking, or residual strain.

For thick-walled cylindrical chambers, yielding occurs first at the inner surface of the chamber walls as pressure is increased (Harvey, 1963, p. 184; Bridgman, 1970). The resulting deformation would cause failure due to seal breakdown and leakage of the contained fluids. From the general stress equations for thick-walled cylinders (Lamé solution), the usual criterion for the material tensile yield strength, \( \sigma_{y_p} \), for a maximum chamber pressure, \( P_{c_{max}} \), is given by:

\[ \sigma_{y_p} = 2P_{c_{max}} \frac{k^2}{k^2 - 1}, \]

where \( k \) is the ratio of the outside to inside diameter. This formula predicts that as \( k \) increases, the required tensile yield strength decreases to \( 2P_{c_{max}} \). Increasing the chamber's wall thickness beyond \( k = 3 \) has limited value. The material chosen for the chamber should have a tensile yield strength greater than \( 2P_{c_{max}} \). The chamber, as designed, has a wall thickness such that \( k \) is slightly greater than 3, and the 303 stainless steel chosen for the chamber walls has a normal tensile yield strength greater than 35,000 psi. The chamber's end-restraints reduce the inner surface stresses since the depth is small, thus providing an additional safety margin.

The necessary strength and thickness of the chamber end-restraints and window support are determined primarily by shear requirements (Harvey, 1963). The average shear stress, \( \tau \), in a circular cross section of thickness \( h \) and radial
distance \( r \) is given by:

\[ \tau = \frac{P_{cr}}{2h} \]  

(5)

Since the maximum shear stress for steel before yield in a tensile test is approximately \( \sigma_{yp}/2 \), the end-restraint or support thickness at a radius \( r \) is given by:

\[ h = \frac{P_{cr}}{\sigma_{yp}} \]  

(6)

The maximum value of \( h \) in the pressure chamber was limited by the required working distances and apertures of the lenses while the minimum value of \( h \) was limited by the window radius and the required internal chamber volume.

There is a wide variety of possible end-closure designs for a constant volume pressure chamber, and many of them have been reviewed by Brahtz (1968, p. 652). The end-closure should be able to contain windows, comply with the working distances of the desired lenses, permit coupling to the pressure pump without interfering with the lenses, and provide rapid access into and cleaning of the chamber. Of the end-closure designs that meet the above requirements, the continuous threaded head requires the shortest chamber length for restraining the end-closure, whereas the bayonet, or breech, mount is the easiest to assemble and disassemble. The bayonet mount was chosen for the chamber because rapid chamber assembly was important for the planned experiments. A Buna-N O-ring (010) seals the pressure chamber. With the gap width kept within 0.001 in and the mating surfaces sufficiently smooth, no back-up rings are necessary. Failure has never occurred at pressures up to 15,000 psi.

The connection to the pressure pump is through standard 316 stainless steel tubing (0.062 in OD, 0.020 in ID, Small Parts Inc., Miami, Fla.) with high pressure stainless steel fittings (High Pressure Equipment Co., Erie, Pa.). The tubing is flexible which allows translation and limited rotation of the chamber on the microscope stage. A 6-in piece of tubing is connected permanently to the chamber and to the rest of the system by a coupling so that the chamber can be cleaned and loaded without the difficulty of removing the connector from the body. Also, this 6-in piece becomes filled with the chamber fluid as the chamber is closed so that the pump hydraulic fluid cannot enter the chamber even at the maximum pressure used.

Several pumps designed to supply oil for hydraulic jacks at pressures up to 10,000 psi are available commercially. Initially, a hand-operated Blackhawk pump (P-76, Blackhawk Mfg. Co., Milwaukee, Wis.), fitted with a standard 10,000 psi Bourdon pressure gauge, was used with the chamber. Because of the small chamber and tubing volume, one stroke of the pump handle could produce pressures as high as 10,000 psi in the chamber within 1 s. A 1-ft section of tubing was filled with inert Kel-F3 fluorocarbon oil to separate the pump hydraulic fluid from the chamber fluids. This section could be removed, cleaned, and refilled before each experiment.

We later realized that the small chamber volume and small tubing volume (about 0.06 ml/ft) would permit the size of the pressure-generating equipment to be reduced considerably. A standard stainless steel high pressure valve (High Pressure Equipment Co., Model 30-11HF4) has more than enough volume displacement between full on and full off (\( \approx 0.1 \) ml) to generate chamber pressures greater than 10,000 psi. Pressure is measured with a low displacement strain gauge pressure transducer (Tyco model AB 10,000, Tyco Instrument Division, Watertown, Mass.) attached to the pressure tubing by an adapter similar to the Tyco model AD-1 having minimal internal volume and machined to receive the 1/16-in OD tubing gland. The resulting system is pictured in Fig. 5.

All component parts are made of stainless steel. Inert Kel-F3 oil, although it is more expensive than hydraulic fluid, can be used throughout the system to transmit the pressure since the total volume is small. About four turns of the valve shaft are required to achieve 10,000 psi. The amount of torque required to rotate the shaft is fairly low, so the valve can be operated easily with one hand if the valve is clamped to the bench. The electrical output of the transducer was read on a large scale 100 \( \mu \)A meter calibrated to read 10,000 psi at full scale using a regulated 5 V source as specified by Tyco. This smaller pressure generating system is considerably more flexible and easy to operate during experiments than was the hand pump.

CRITERIA AND DESIGN BASED ON CONSIDERATIONS OF THE OPTICAL PROPERTIES OF THE CHAMBER WINDOWS

The success of any chamber designed for use with phase-contrast or polarized-light microscopy depends primarily on the optical characteristics of...
FIGURE 5 The pressure chamber with equipment for generating and measuring pressure. The assembled chamber is installed in its temperature-control stage and connected to the valve pump and electrical pressure transducer. Following are the part numbers of the stainless steel fittings obtained from High Pressure Equipment Co. A HIP no. 15-2AMI; B HIP no. 15-21AF1; C HIP no. 30-11HF4; D HIP no. 15-21AF1HM4. E is the Tyco transducer and adapter described in the text.

the windows. Perfect image formation occurs in a microscope when light rays emanating from an object arrive at the image point with no difference in the optical path lengths. The total optical path length of a single ray of light is the summation of the products of the refractive indices of the media through which the light ray passes and the distance the light ray travels through each medium. Differences in optical path lengths are introduced by the window geometry, the window material, and the magnitudes and distributions of stresses in the window.

When a glass plate, such as the chamber window, is placed between the specimen and the lens, the optical path lengths of high aperture rays are different from the optical path lengths of low aperture rays because the high aperture rays travel a longer distance through the glass. This produces spherical aberration which is correctable by choosing appropriate objectives as mentioned previously.

A material in which the refractive index differs for different directions of electric vibration is said to be anisotropic or birefringent. The length of the optical path traversed by a wave front passing through such a material will depend on the direction of wave propagation (of the light ray) through the material and the direction of vibration of the wave's electric vector (Born and Wolf, 1965, p. 673). In addition, when unpolarized light passes through an anisotropic material it may travel as two components having mutually perpendicular electric vectors and two different velocities. The same thing can happen with polarized light if the electric vector is not aligned with one of the principal directions in the material. Consequently, the two component wave fronts will traverse different optical path lengths and will become altered in phase. This difference in optical path is called "birefringence retardation" (BR). Anisotropy in a material may be an inherent result of the material's molecular structure or the result of a stress-induced change.

For image formation with light microscopy, in general, the optical path lengths over a given wave front of light should not differ by more than a quarter of the wavelength of the illuminating light (Longhurst, 1967, p. 354). For adequate image contrast in phase-contrast microscopy, the difference in optical path lengths for a wave front propagating through the system should not differ by more than one-eighth of a wavelength (Slayter, 1970, p. 201). However, with polarized-light microscopy, to detect birefringence as weak as 0.5 nm (not unusual in the mitotic apparatus), the optical path lengths of two mutually perpendicular component wavefronts should, on the average, not differ by more than about 0.001 of a wavelength.

Although crystalline materials such as quartz or sapphire are commonly used for windows in pressure chambers because of their high strength (Hamann, 1957; Pugh et al., 1963; Robertson, 1963), they are not suitable here because they are highly birefringent. Annealed, strain-free optical glass has negligible refractive index anisotropy, so is a better choice for the window material. With no inherent refractive index anisotropy in a glass window, the major concern is stress-induced anisotropy which is related both to the photoelastic properties of glass and to the magnitudes and distribution of stresses in the windows.

Photoelasticity

When an isotropic material, like glass, is subjected to stress, a change occurs in the material's refractive index. This change is proportional to the stress, but different for different directions, producing optical anisotropy or "stress birefrin-
gence." More specifically, for any point of stress in an isotropic material, the axes of the material's refractive index ellipsoid are coincident with the axes of the principal stress ellipsoid at that point. The magnitudes of the axes of the refractive index ellipsoid differ from the isotropic value proportionally to the stress, but the constants of proportionality are different for different directions. The absolute stress optical coefficients, \( C_1 \) and \( C_2 \), are the proportionality constants for refractive index changes: \( C_1 \) for the change produced in the direction of a principal stress; and \( C_2 \) for the change produced in the two perpendicular directions by the principal stress. The relative stress optical coefficient \( C \) equals \( C_1 - C_2 \). For plane stress \( P \) measured in psi, the amount of BR, measured in nm, through a distance \( h \) (mm) normal to the plane stress is given by:

\[
BR = \left( \frac{Ch}{147} \right) (P_1 - P_2),
\]

where \( P_1 \) and \( P_2 \) are the magnitudes of the principal stress components of \( P \).

The stress optical coefficients are measured in Brewsters (B), defined as the magnitude of the stress optical coefficient for which a simple stress of one bar (\( \approx 1 \) atm, \( \approx 14.7 \) psi) produces a relative retardation, or change in optical path length, of \( 10^{-1} \) nm for light passing through a thickness of 1 mm in a direction normal to the stress (Coker and Filon, 1957, p. 185). The relative stress optical coefficient of most commercially available glass is about \( +3 \) B with the absolute stress optical coefficients \( C_1 = -1 \) B and \( C_2 = -4 \) B (Coker and Filon, 1957). A more detailed discussion of photoelasticity and its consequences is given by Salmon (1973), Coker and Filon (1957), and Jessop and Harris (1960).

To achieve adequate image contrast, the maximum optical path length differences must not exceed the values stated earlier. From these limiting values and knowledge of the relative and absolute stress optical coefficients of the glass used, as well as window thickness (\( h \)), the average maximum permissible stress anisotropy may be calculated from equation (7). For the windows employed (\( h \approx 1.75 \) and \( C \approx 2.75 \) B), the phase-contrast criterion (\( \lambda/8 \)) is reached at 1,752 psi stress difference, while the polarized-light criterion (\( \lambda/1,000 \)) would be reached by an average stress difference of only 16.7 psi through one window.

While the permissible stress difference for phase-contrast microscopy is not large, the permissible stress difference for polarized light is extremely small. Consequently, the successful use of the chamber, especially with polarized-light microscopy, depends on the actual stress distribution in the windows produced by pressurization of the chamber.

**Optical Performance**

Fig. 6 shows that for phase contrast microscopy there is no appreciable image deterioration at pressures as high as 10,000 psi. The only significant aberrations produced by the chamber result from vignetting near the edge of the field due to the chamber port aperture. No vignetting, though, occurs within a central area 1 mm in diameter.

Fig. 7 shows that image contrast with polarized-light microscopy is affected by increased pressure. As pressure increases, the background light intensity also increases due to increased stress BR in the window. Up to 4,000 psi, the effect on the quality of the image is slight; above 4,000 psi, the effect is more significant. Nevertheless, the 0.7-nm retardation area, for which the compensator was set to produce maximum contrast, is still visible at 10,000 psi.

The best image contrast was obtained for specimens near the center of the window since the intensity of the background light becomes increasingly strong away from the center and from the axis directions of the analyzer and polarizer. Up to 4,000 psi, a good image is produced within a central area about 1 mm in diameter. At 10,000 psi, a central area about 0.3 mm is usable. With objectives having a numerical aperture higher than that of the Nikon 10x objective used for the photographs in Fig. 7, the image quality is not as good. For experiments concerning spindle assembly the image quality in polarized light is perfectly acceptable with the low resolution objectives because of the relatively large spindle dimensions and because spindle disorganization and total disappearance of spindle BR generally occur at a pressure below 4,000 psi.

**Analysis of Stress Distribution and Its Effect on Image Quality**

The overall image quality in polarized light is remarkably good, considering the severe restrictions on permissible stress differences in the windows. Although the magnitudes of stress in the windows can be higher than the chamber pressure, the magnitudes of stress anisotropy are low, due to

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An accurate determination of stress-induced refractive index anisotropy in the windows is complicated. A detailed assessment of the stress distribution is presented elsewhere (Salmon, 1973); only the major features will be presented here. Four primary factors contribute to the stress distribution: the circumferential loading of the window by the chamber pressure; the transferral of the normal chamber pressure from the lower window surface to the window seat area; the shearing stresses near the port rim; and the bending of the window into the port opening.

Loading of the window circumference with the chamber pressure produces isotropic lateral compressive stress in the window equal in magnitude to the chamber pressure. For glass, this produces a positive uniaxial refractive index ellipsoid whose major axis is parallel to the window's radial axis of symmetry. Under this stress condition, the window behaves as a positive uniaxial crystal having its optic axis aligned with the chamber symmetry axis. When the specimen is located at the axis of symmetry, the amount of BR produced by the circumferential stress for a ray with an inclination angle \( \theta \) to the symmetry axis is given by:

\[
BR (\theta) = \frac{(CH \cos \theta)}{147 \cos \theta},
\]

where \( h/\cos \theta \) is the distance in millimeters traveled through a window of thickness \( h \) at a ray angle \( \theta \), and \( P_c \) is the chamber pressure measured in psi (Wahlstrom, 1960, p. 167; Salmon, 1973). This is a radially symmetrical positive BR which is zero for rays along the axis of symmetry but increases progressively with higher aperture ray angles.

The stress distributions and optical influence of the other three factors cannot be determined precisely. Because of the cylindrical symmetry of the window, however, they also will contribute a radial BR which increases with ray aperture angle as described by equation (8) and with radial distance from the axis of symmetry (Salmon, 1973).

A quantitative assessment of the window stress distribution was determined experimentally with the polarization microscope as illustrated by the objective back aperture photographs in Figs. 8 and 9. Light rays diverging from the specimen region with an angle \( \theta \) to the optic axis are focused in the objective back aperture at a radial distance \( d, (\theta) \) given by:

\[
d, (\theta) = \frac{a \sin \theta}{N A_{OBA}},
\]

where \( a \) is the radius of the objective back aperture and \( n \) the refractive index of the window. As seen in Fig. 8, with increasing pressure the birefringence remains zero for rays along the central axis of the window and for rays whose inclination is parallel to either the analyzer or polarizer directions. This
FIGURE 7 Photomicrographs of the birefringent surface of a cheek squamous epithelial cell with increasing pressure in the chamber. The microscope was equipped with a strain-free rectified Nikon 10x objective, and a strain-free rectified Leitz UM 20x objective as a condenser. Compensator setting for the upper row of pictures was +3 nm and for the lower, -5 nm. Analyzer and polarizer directions are at 45° to the margins of the plate. The approximate BR (nm) for various regions of the cell surface, which does not change with increasing pressure, is indicated in the accompanying sketch. The specimen was mounted against the inner surface, near the center, of the objective window of the chamber. Scale indicates 10-μm intervals.
produces the dark cross patterns since at a given pressure the light rays become more birefringent as the ray aperture angle increases and as the radial direction of the ray approaches 45° to the polarizer and analyzer directions. The dark cross pattern does not change significantly as the chamber is rotated, which indicates a symmetrical radial stress distribution. At higher pressures, the dark cross narrows and becomes more defined because the stress anisotropy increases progressively for the higher aperture rays. A slight unidirectional lateral stress, about 2 nm at 10,000 psi, was present but easily cancelled with the compensator.

As illustrated in the photographs in Fig. 9, the addition of a known amount of compensator BR produces dark fringes in the back aperture. The fringes correspond to rays whose BR is equal to but opposite in sign to the BR added by the compensator. The variation in stress BR with aperture angle (Fig. 10) was determined by measuring the radial position of the dark fringes at 45° to the analyzer-polarizer directions as a function of the compensator BR (Jessop and Harris, 1960, p. 66). On the basis of symmetry considerations, each window contributes about half the measured BR.

The window BR is radially positive with respect to the window's central axis, proportional to the chamber pressure, and increases slowly at low apertures and progressively faster at higher apertures as shown in Fig. 10. The expected BR contributed by the circumferential loading stresses, as determined from equation (8), is also plotted in Fig. 11 for C = 2.75 B and a combined window thickness of 3.5 mm. The measured BR has the same form as equation (8), but it is approximately 1.4 times higher. This suggests that the stress distribution due to the circumferential loading of the window by the chamber pressure contributes about 70% of the BR, while the bending and shearing stresses and the stresses due to the vertical loading together contribute only 30%.

**DESIGN FEATURES BASED ON CONSIDERATIONS OF EXPERIMENTAL USE**

Since the chamber is designed for experiments in which pressure is to be the major variable, we need to know what transient temperature changes occur when the chamber is pressurized. The experimenter must also be able to control the equilibrium temperature. Furthermore, the chamber should
allow rapid, efficient mounting of specimens and cleaning.

**Pressure-Induced Transient Temperature Shifts**

If a fluid is compressed adiabatically (with no heat transfer) there would be a temperature rise given by:

\[ T = \left( \frac{T}{\rho C_p} \right) P, \]  

where \( T (\text{K}) \) is temperature, \( \rho \) is density, \( \beta \) is the coefficient of thermal expansion, and \( C_p \) is the material heat capacity (Zemansky, 1957, p. 249).

In general, the temperature change for a material is proportional to the pressure change. Because of the chamber's small internal volume and large internal surface-to-volume ratio, one would expect the duration of temperature changes to be short.

A small thermistor bead (Fenwall model GB35J1, Allied Electronics, Chicago, Ill.), less than 1% of the chamber volume in size and having a time constant of 0.3 s, was placed inside the chamber and connected to a bridge circuit. The output was read off a calibrated scale from a high speed chart recorder (Brush Model 220, 3 dB down at 125 Hz, Gould Inc., Cleveland, Ohio). Transient changes in distilled water, seawater, and the fluorocarbon oils Kel-F3 and Kel-F10, were determined. As an example, Fig. 11 shows transient changes in the temperature of Kel-F10 oil for application and release of 6,000 psi. Since pressure affects the thermistor's output, the pressure-induced shift must be subtracted from the thermistor output reading to obtain the shift induced by temperature (ZoBell, 1959). For all the fluids tested, the time constant for re-equilibration was about 7 s. The peak magnitude of the temperature pulse depended on the fluid and was found to be proportional to the pressure, as expected from equation (10). The proportionality constant for distilled water and seawater is 1°C/10,000 psi, and for the fluorocarbon oils it is 2.7°C/10,000 psi. These values are about two-thirds the values expected from equation (10) and the material constants. This difference is due primarily to the rapid heat transfer which begins immediately after the pressure change and, to a smaller degree, to the 0.3-s time constant of the thermistor.

**Stage Holders and Temperature Control**

Fig. 12 shows a stage holder for the chamber which fits under the spring clips of most microscope stages. A removable flange was used to fit the holder directly over the circular stage support of the Nikon Model S microscope used in the experiments reported in Salmon 1975 a-c.

The temperature of the chamber can be controlled within 0.15°C by passing water from a temperature-regulated water reservoir at about 100 ml/min through a water jacket encircling the chamber. The time constant for shifting the internal temperature was about 45 s. A brass cup was

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**Figure 10** Window stress BR as a function of chamber pressure and inclination angle of the ray to the axis of symmetry. The microscope was focused at the center of the chamber, midway between the inner surfaces of the windows. The measurements were obtained from photographs of the objective back aperture as explained in the text. The solid lines represent the estimated BR contributed solely by the circumferential loading of the chamber windows by the chamber pressure as calculated from equation (8).

**Figure 11** Transient temperature changes induced by pressurization of the chamber filled with Kel-F10 fluorocarbon oil exemplified for 6,000 psi. The curve was traced from the original chart recording. Pressure was applied with a Blackhawk hand pump. Application of pressure required about 1 s, but the release of pressure by opening a valve was nearly instantaneous. Temperature was measured with a bead thermistor (time constant 0.3 s) placed inside the pressure chamber as described in the text. The same results were obtained whether or not the chamber was immersed in a water bath.
FIGURE 12 Temperature-controlled stage holder for the pressure chamber. 0.25-in ID Tygon tubing was used to connect the stage to a temperature-regulated water reservoir. A 0.25-in OD copper tubing; B brass cup; C Lucite base; D air duct. The brass cup is slit next to its base through two-thirds of its circumference, to provide a snug spring fit with the chamber inside. Scale: 1 cm.

machined with an internal diameter equal to the external diameter of the pressure chamber. A piece of quarter-inch OD copper tubing was soldered about its circumference and the cup split, as shown in Fig. 12, so that the chamber fitted snugly inside. The brass cup was attached to a Lucite base which had a circular channel for blowing dry air across the lower chamber window to prevent fogging at low temperatures. A small tube attached to the objective was used to pass dry air across the upper chamber window surface. The water jacket temperature was monitored at both ends of the copper tube, with Type 401 thermistors and a Model 43TD bridge (Yellow Springs Instrument Co., Yellow Springs, Ohio).

**Specimen Preparation and Cleaning the Chamber**

Most standard slide-cover slip preparation techniques that do not require entrapped air spaces are easily adapted to the chamber. If the objective working distance is not restrictive, the specimen is best mounted against the condenser window because it is easier to clean. There was little difference in the optical performance of the chamber with the specimen mounted on either the upper or lower window.

Depending on cell type, two preparation techniques have proven most convenient. In one, a small drop (~10 μl) of medium containing cells is placed on the center of a clean dry window between two small ridges of silicone grease (Dow Corning Corp., Midland, Mich.). A 2 mm × 4 mm cover slip fragment is immediately set in place and gently compressed to squeeze out all entrapped air and to flatten the cells slightly, thus holding them in place. Fresh specimen medium is added as quickly as possible to prevent effects of evaporation. Cells can be repositioned in the field by gently moving the cover slip fragment. The chamber is then filled completely with specimen fluid to eliminate problems caused by entrapped air, and closed.

Inert fluorocarbon oils are also useful to hold and flatten cells against the window, particularly insect spermatocytes and plant endosperm cells. Preparation is essentially the same as a normal slide-cover slip preparation (Forer, 1965), except that the cover slip is not required. When the chamber is filled with the oil and closed, the other window serves the same purpose as the cover slip.

The chamber was best cleaned with Ivory soap and rinsed repeatedly with tap water, then distilled water. The detergent Alconox appeared to cause the O-rings to swell and so was not suitable. Alcohol was useful as a solvent for the fluorocarbon oils. During the cleaning process, care must be taken to avoid scratching the windows. To clean the outer window surfaces, it is necessary to assemble the chamber filled with fluid in order to prevent dislodging the windows.

**FURTHER IMPROVEMENTS**

In its present form, the chamber still has several features which could be improved. If a stronger stainless steel were used in the chamber constructions, such as maraging stainless steels, the external chamber depth could be reduced by as much as a factor of four, while still maintaining the preferred chamber geometry, internal volume, and window size. This would allow higher aperture objectives and condensers with shorter working distances to be used.

Although the optical performance of the cham-
ber in phase contrast is entirely satisfactory for pressures to 10,000 psi, the performance in polarized light requires improvement to achieve comparable resolution in weakly birefringent specimens over the same pressure range. Modifications of the window geometry or the use of a radial-birefringent compensator system may prove useful. The most direct approach, however, would be to use a window material with a lower stress optical coefficient. The stress optical coefficient of flint glasses is about 3 B for a lead oxide content below 40%. As percentage of lead oxide increases, the stress optical coefficient decreases, becoming negative above 75% lead oxide (Coker and Filon, 1957, p. 218; Waxler and Napolitano, 1957). Geffcken and Jacobsen (1962) have measured a value of C = -0.004 B for Pockels glass (75% PbO, 24% SO4, 1% K2O) at a wavelength of 500 nm. However, Pockels glass would have to be specially made for the windows as it is not a standard product.

The microscope pressure chamber, as it stands now, provides for the first time a means for observing directly internal structural detail in living cells under pressure. While not without limitations, the chamber can be used conveniently with any standard microscope system and provides excellent image quality for low resolution polarized-light and moderate resolution phase-contrast microscopy.

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