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A 77-K cold stage for Raman microprobes and optical microscopy

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A 77-K cold stage has been developed for spectroscopic measurements in an optical microscope. This stage eliminates fogging of the optical windows observed in an earlier design by adding vacuum jackets around the liquid nitrogen transfer lines and by maximizing the sample-to-environment distance without sacrificing optical imaging power. Increased maneuverability of the cold stage is achieved by using flexible stainless-steel liquid-nitrogen transfer lines. In our application, the sample in the cold stage is illuminated with a focused laser beam and vibrational resonance Raman scattering spectra are recorded. We have used this Raman microprobe system to measure the Raman scattering from a variety of individual rod photoreceptor cells. This work has provided new information about the mechanism of wavelength regulation in color vision.

INTRODUCTION

Lasers and highly sensitive intensified multichannel array detectors have spurred the current renaissance in biological optical microscopy. New techniques include confocal fluorescence microscopy,[1] three-dimensional optical microscopy,[2] and the Raman microprobe.[3–9] However, the intense optical illumination associated with lasers can produce local heating, deleterious photochemical intermediates, and photobleaching, which place fundamental limits on these techniques. In our own case we were interested in *in situ* studies of photoreceptor pigments which are exquisitely light sensitive and can only be studied with a Raman microprobe by cooling the samples to 77 K. At low temperatures, a photostationary steady-state mixture of the pigment and its primary photoproduct are produced since the thermal decay of the photoproduct is blocked.[10] Therefore, we have developed a cryogenic cold stage for our Raman microscope.[11]

Designing a cryogenic stage for optical microscopy of biological systems presents several unique problems. The cold substrate and sample should be isolated from the environment to minimize heat transfer. However, the optical necessities of the microscope prescribe that the distance between the sample and focusing lens (and condenser lens) be as small as possible for the greatest magnification, resolution, and light-gathering ability. Biological samples present additional difficulties since they are only stable in aqueous solutions. Thus, any thermal insulation system used in the cold stage must be compatible with aqueous samples. Although a number of microscope cold-stage designs have been reported,[12–14] none have addressed the special handling requirements needed for aqueous biological samples. Most of these designs have differed only in their methods of cooling.

We have previously reported a prototype cryogenic microscope stage (see Fig. 1) for use with aqueous biological samples in a Raman microprobe.[4,7,8] Our original cold stage design needed improvement in the areas of thermal isolation, maneuverability, mechanical stability, and condenser access. In this article, we present an improved design which solves these problems.

I. CRYOGENIC STAGE DESCRIPTION

It is useful to briefly describe the operating concept of our stage before discussing the details of the design. First, a small drop of an aqueous sample is loaded onto a sapphire window. The sample is then covered with a 0.1-mm cover slip and compressed to $\sim 50\ \mu$m thickness. The substrate is cooled below 0 °C while the chamber is purged with dry nitrogen gas. Once the sample is frozen, the cold stage is evacuated and the substrate cooled further to 77 K. We have designed the cold stage to cool as rapidly as possible and to remain frost-free and mechanically stable. The first requirement ensures a low-temperature aqueous glass of good optical quality. The other criteria are necessary for collection of data over long periods of time.

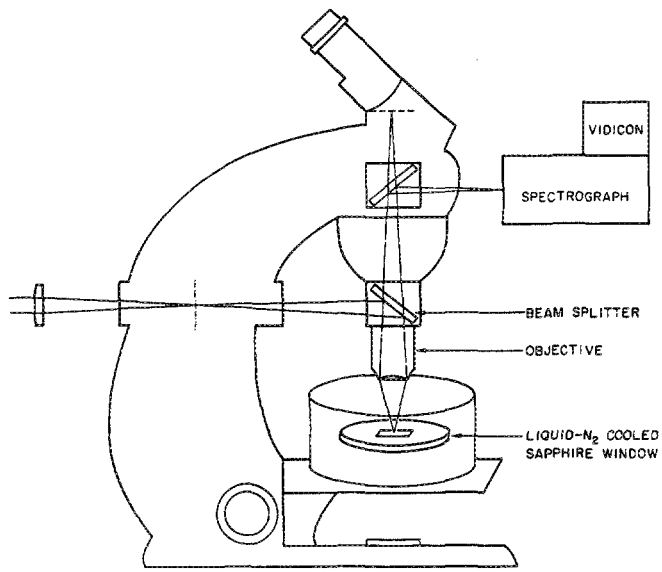

FIG. 1. Schematic of the Raman microprobe and liquid-nitrogen cold stage.[11] A Zeiss universal microscope is coupled to a subtractive dispersion spectrograph[16] and multichannel detector. The position of the cold stage is controlled with a conventional Zeiss microscope translation stage. Zeiss LD-epiplan objectives focus the laser beam on the photoreceptors and collect the back-scattered Raman light.

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The design of the body of the stage is shown in Fig. 2. The cold stage is constructed primarily of stainless steel. The body contains the cold tip, transfer lines, and outer ports for the vacuum, nitrogen gas purge, and thermocouple. Cryogenic temperatures are achieved by flowing liquid nitrogen from a 50-ℓ dewar through a double-walled, vacuum-insulated transfer line (Precision Cryogenic Systems, Indianapolis, IN). The transfer line is 1.0 m long and is composed of corrugated, double-walled stainless steel (0.38 in. i.d., 1 in. o.d.). The liquid nitrogen inlet line is constructed of 3.2 mm o.d., 0.5 mm wall stainless-steel tubing, insulated with vacuum jackets constructed of 12.7 mm o.d., 0.9 mm wall stainless-steel tubing. Stainless-steel washers 1 mm thick are silver-soldered to the vacuum jackets at either end to support the liquid-nitrogen lines. The vacuum port is connected to a vacuum manifold with rubber vacuum tubing to minimize coupling of vacuum pump vibrations to the stage.

The cold tip consists of a sapphire window in a liquid-nitrogen-cooled brass block. Sapphire was used since its thermal conductivity is 20-times higher than that of quartz and ~3800 times higher than that of glass at liquid nitrogen temperatures.[15] The sapphire window (12.7 mm diam., 1 mm thick) is fastened to the 2-cm × 2-cm × 5-mm brass cold tip by an indium gasket. The liquid nitrogen flows around the brass cold tip through a 0.13-in. channel indicated by the dashed line in Fig. 2. Once the liquid nitrogen has passed through the cold tip, it is discharged to a waste dewar. The temperature of the sample is monitored by a calibrated copper-constantan thermocouple attached to the brass tip.

The dimensions of the cold stage near the sample are the most critical design parameters. The extremely small distances needed between the sample and the objective and condenser lenses, coupled with the need to thermally isolate the sample are two critical constraints. Figure 3 shows our optimization of these parameters. To achieve maximum thermal isolation of the sample, we used a long working-length condenser lens (Zeiss aplanatic NA 0.63, working length 7–11 mm) and long working-length objectives (Zeiss LD-epiplan 16×, NA 0.3, working length 3–7 mm and 40×, NA 0.6, working length 3–4 mm). These lenses allowed the top optical window to be placed 1 mm above the working surface of the sapphire window and the bottom window to be 6 mm from the sample (Fig. 3). The objective was typically 1 mm above the top optical window when the sample was focused. The condenser lens was typically within 1 mm of the bottom window. The objectives are corrected for 1-mm thick cover slips, requiring that the optical windows be 1-mm thick. The use of a well to accommodate the microscope objective (see Fig. 3) simplifies the mechanical design tolerances of the rest of the dewar and also increases the thermal isolation of the cold tip. Thermal contact between the liquid nitrogen system and the ambient environment occurs through the points where the vacuum jacket is connected to the liquid-nitrogen line (Fig. 2). Since these points are (thermally) distant from the body of the dewar, this minimizes the cooling of the body and concomitant fogging of the windows.

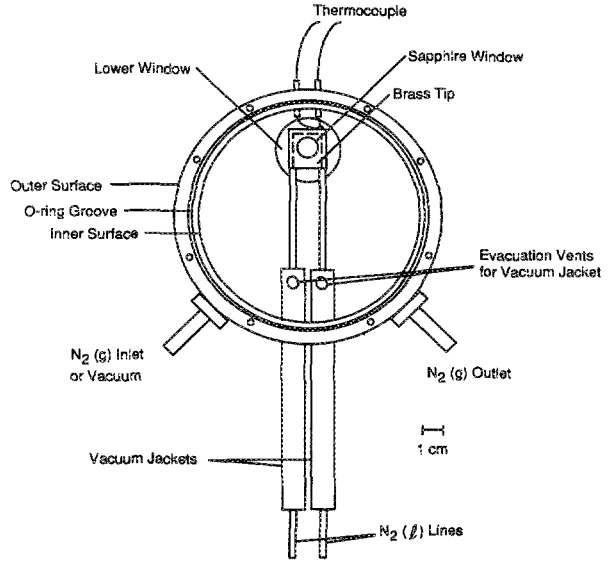

FIG. 2. Top view of the body of the liquid-nitrogen cold stage. The sample is loaded onto the sapphire window which is in thermal contact with the brass tip. Liquid nitrogen flows from a dewar and transfer line, through the brass tip, and out to a waste dewar. The two additional ports are for the vacuum and nitrogen purge.

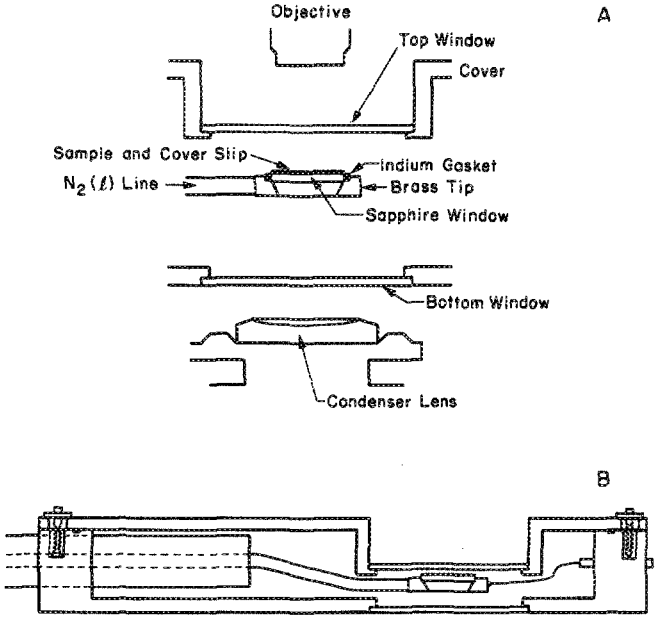

FIG. 3. Exploded (A) and assembled (B) cross-sectional views of the cold stage. The gap between the sample and the top window is 1 mm, and the distance between the sample and the bottom window is 6 mm. The windows and substrates are 1 mm thick.

II. OPERATION

The operation of the cold stage can be broken down into three steps: (1) Before cooling, the transfer line, liquid-nitrogen lines, and stage interior are purged with dry-nitrogen gas for approximately 1/2 h to remove water vapor; (2) Then, ~50 $\mu\ell$ of the aqueous sample is loaded onto the sapphire window and covered with the cover slip. The cover slip is physically pushed down to achieve a minimum sample

thickness of $\sim 50\,\mu$m. The top cover is then put on while the interior of the stage is purged with dry-nitrogen gas; (3) At this point, the liquid nitrogen reservoir is pressurized with nitrogen gas at 10 psi, and liquid nitrogen is forced through the cold stage transfer lines to cool the sample. When cooled rapidly to below 0 °C, the thin aqueous sample produces a clear, crack-free glass with no visible evidence of crystalline ice. As the temperature of the sample passes through -10 °C, the nitrogen-gas purge is stopped and the chamber is evacuated to a final pressure of ~ 1 mTorr. Once the temperature reaches ~ 77 K, the liquid nitrogen flow is adjusted to its minimum value to keep the temperature constant. Fifty liters of liquid nitrogen were required for ~ 3 h of operation. Of this, only 10–15 ℓ are actually used in the cooling process and the rest is dumped in the waste dewar. In all experiments, the minimum temperature reached on the brass tip was 77 K.

III. EXPERIMENTAL RESULTS

The operating characteristics of the cold stage are as follows. The cooling rate is sufficiently fast to produce a glass of good optical quality. Initial cool-down to -10 °C occurs in $\sim$20–30 s. Once the cold stage is evacuated, the cooling rate is significantly enhanced. To additionally cool the stage to 77 K requires only 1.5 min. The severe fogging problems encountered with the earlier design were absent with this stage. Some fogging was seen on the optical windows on the most humid days because of the close proximity of the cold tip to these windows. This problem was solved by simply blowing dry-nitrogen gas over these windows. The flexible, corrugated stainless-steel transfer line allowed the stage to be easily translated several inches and it was mechanically stable ($<2\,\mu$m motion per 15 min).

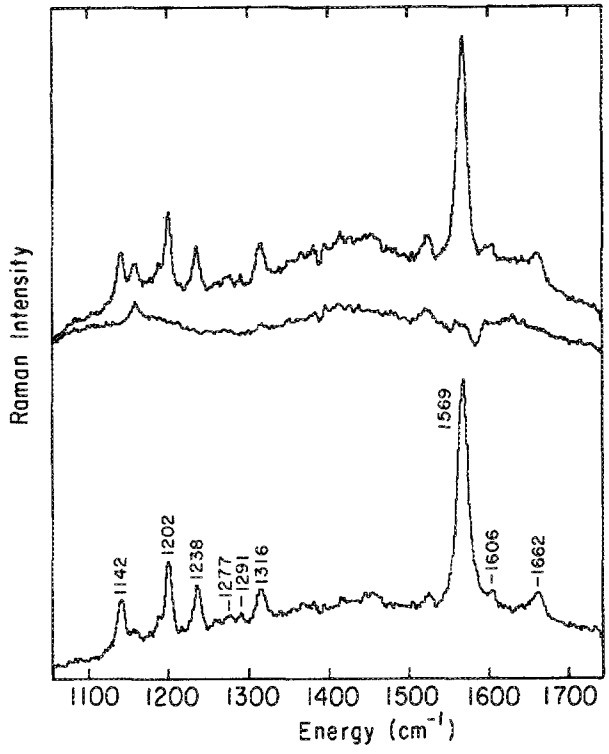

FIG. 4. Resonance Raman spectra of the visual pigment found in the green rod outer segment from the toad, *Bufo marinus*.[11] Raman scattering was excited by focusing a 4.5-mW, 514.5-nm laser beam with a Zeiss 40× objective onto a single 10-μm diam green rod outer segment frozen at 77 K in Ringer's buffer. The top spectrum is the sum of four 4-min accumulations from the green rod outer segment. The middle spectrum is the sum of four 4-min accumulations from the buffer next to the cells taken under identical conditions. The bottom difference spectrum is the baseline-corrected resonance Raman spectrum of the pigment.

An illustration of the use of the cold stage in the Raman microprobe is given in Fig. 4. A preparation of rod outer segments from the toad *Bufo marinus* was frozen in the cold stage as described above. A 4.5-mW laser beam at 514.5 nm was focused on one 10×50-μm "green" rod outer segment and the back-scattered Raman light was recorded with the spectrograph and multichannel detector shown schematically in Fig. 1. Four integrations of the Raman spectrum were obtained and averaged. Over the 16 minutes required to obtain the Raman spectra, no significant decrease in the intensity of the peaks was observed, indicating that the alignment of the sample and transparency of the optical windows were constant. The sample glass was of good optical quality, as evidenced by the high signal-to-noise ratio of the Raman spectrum and low background scattering.

These data provide new information about the molecular mechanism of wavelength regulation in color visual pigments.[11] In particular, the 1662-cm^{-1} Schiff base C=NH stretch demonstrates that the retinal prosthetic group in the "green" rod pigment has a protonated Schiff base linkage to the protein. Furthermore, the frequencies of the skeletal modes in the 1100–1300-cm^{-1} fingerprint region indicate that there are no additional protein-chromophore perturbations that affect the absorption maximum. Thus, the 440-nm absorption maximum of this blue-absorbing visual pigment is determined solely by protonation of the Schiff base linkage to the protein.[11]

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