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## **CALI protocol**

The basic principle of the Chromophore Assisted Laser Inactivation (CALI) technique is the interaction of photons with chromophores able to absorb these photons and subsequent photochemical production of active species (mostly reactive oxygen species (ROS)). These active species diffuse from the chromophore to the very proximate region and react with the nearest available target. If this is an introduced protein labeled with such a chromophore, one or more functions of the protein may be inactivated. The CALI technique was pioneered by Dan Jay and developed further by other researchers.

### **CALI chromophore**

The first step is to attach the appropriate chromophore to the protein of interest. Several have been employed as sources of CALI action, thus far. Jay originally employed malachite green (1). More recently EGFP (2,3); FIAsh (4) and ReAsH (5) have all be used. The choice of chromophore determines the labeling or expression procedure required.

When choosing a chromophore for CALI one should consider:

1. Absorption/excitation spectra of the dye, which will determine the excitation wavelength of the illumination source
2. Quantum efficiency of free radical production upon irradiation dictates the inactivation efficiency
  - -Fluorescein, FIAsh, ReAsH – high efficiency (low excitation power – high inactivation)
  - -Malachite green, EGFP – low efficiency
3. Ease of attaching chromophore to the protein of interest and specificity of added dyes.

A potential issue for exogenous chromophores is unspecific binding. For example, FIAsh which is engineered to bind to tetracysteine motifs expressed in target protein, may bind cysteine-rich domains in proteins other than the target resulting in some non-specific inactivation (6,5). To check the specificity of staining in your cell type, stain wild type cells not expressing tetracysteine motifs. If no staining is detected, CALI can be

performed with confidence provided other controls listed below are negative.

4. Cell tolerance to the labeled protein. Some staining procedures for exogenous dyes may involve the use of harmful components which can interfere with cell behavior. For example, in using the arsenic containing dyes, FIAsh and ReAsH, EDT antidote in micromolar concentration must be added to minimize binding to endogenous cysteine pairs or lipoamide cofactors. Such an unspecific binding could cause toxicity and/or nonspecific labeling (7,8). Higher, millimolar concentrations of EDT can reverse the FIAsh staining.

### **Light sources**

The most common light source in the fluorescence microscopy is mercury lamp outfitted with a set of excitation filters and it can be used for CALI. This can be used to irradiate a whole field of view or restricted to only a part of the cell. There are also reports of normal, low power halogen lamps being used in CALI experiments (9).

Lasers are commonly used as an irradiation source in CALI technique and in many ways are the preferred source. They emit monochromatic light and can be focused to very small spots on the specimen (<1 micron).

Desirable features of a CALI light source include:

- high level of monochromaticity
- regulated level of the output power
- ability to change size of the irradiated area using diaphragms or by focusing and defocusing

### **Sample CALI protocol**

The following is an example of a CALI protocol as described in Rajfur et al (2). For cell irradiation, the light beam from a Spectra Physics 164 (Spectra-Physics Laser Incorporated, Mountain View, CA) argon ion laser (488-nm line, 500 mW of beam power at the laser head) was focused to a 2.2- $\mu$ m diameter spot ( $1/e^2$  diameter) using a 100 $\times$ Phase 3 Neofluor objective mounted on a Zeiss Axiovert 10 inverted microscope. Beam power dropped to 40 mW at the object plane because of optical losses, measured by positioning the sensor of a laser power meter (model FM with LS 10 head; Coherent, Auburn, CA) at or near the specimen plane. Irradiation time was 100 ms, resulting in 4-mJ dose of irradiation energy for CALI. The initial beam was split into two. One beam,  $10^4$  times weaker

than initial one, was used to visualize the irradiation spot without causing appreciable damage to the cell. The second beam was the full power CALI irradiation beam, which was blocked by a fast Uniblitz (Vincent Associates, Rochester, NY) shutter and opened only during the irradiation period. Pre-and post-CALI images were acquired with a Hamamatsu 4880 CCD camera (Hamamatsu Photonics Corp, Bridgewater, NJ) and processed with Metamorph imaging software (Universal Imaging Corp. West Chester, PA).

## Controls

Controls should be designed to eliminate non specific photodamage and to as much as is possible to ascribe the loss of function to the 'CALI'ed' protein. Thus, the biological effects of irradiation in the absence labeled or fusion proteins should be assessed. Also the effects of irradiation of irrelevant labeled or fusion proteins should be examined.

It is important to realize that the CALI technique is still relatively new and, as such, it is still being developed as a specific loss-of-function technique.

## References

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