In vitro EGFP-CALI comprehensive analysis

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To determine the mechanism of EGFP-CALI

INTRODUCTION

1. CALI background

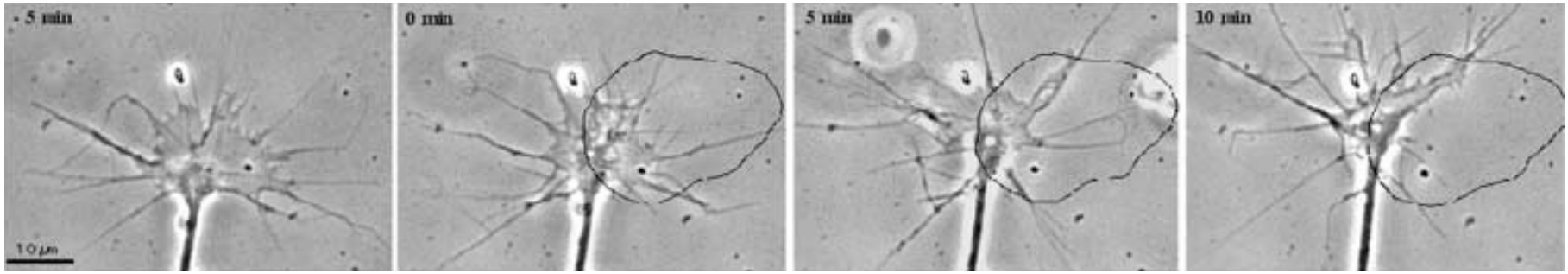
- Chromophore-assisted laser inactivation (CALI) is a technique that selectively inactivates proteins of interest to elucidate their in vivo function. [1]
- In brief, the protein to be inactivated can be targeted in three ways: 1, antibody conjugated with fluorescein; 2, EGFP fusion protein; 3, expressed as a protein that will bind FlAsH [2].
- Mechanism: Inactivation of protein by CALI is mediated by free radicals upon dye excitation (not photothermal denaturation) [3].

2. *In vivo* CALI experiments

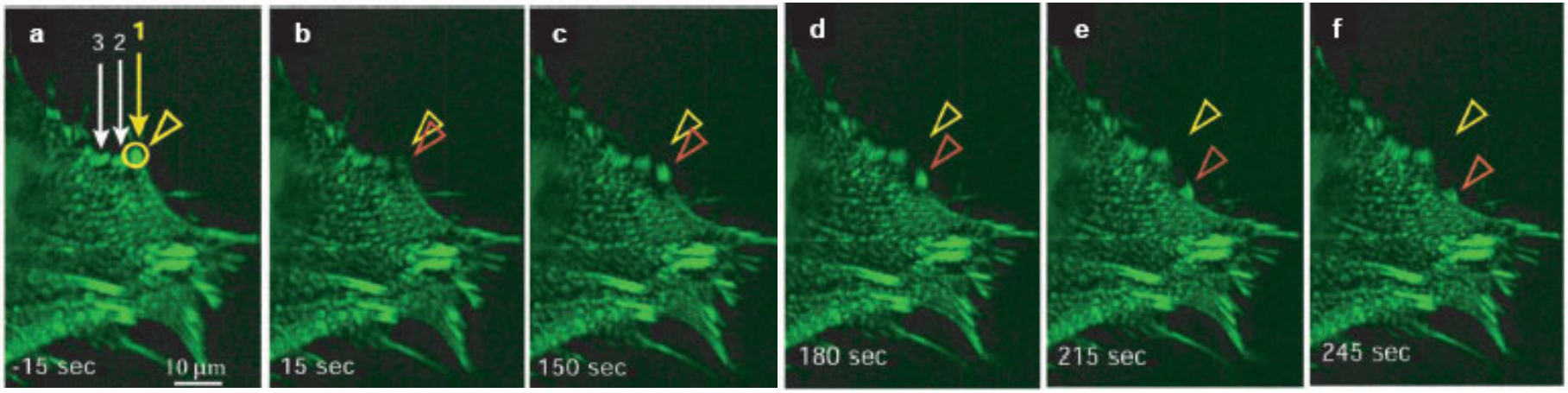
Using CALI to inactivate myosin II, which were labeled with malachite green, in the entire chick dorsal root ganglion, Diefenbach *et al* showed a reduction in neurite out growth

[1] Jay, D.G. (1988) *Proc.Natl.Acad.Sci. USA* 85, 5454-5458
[2] Griffin, B.A., Tsien, R.Y., etc. (1998) *Science* 281, 269-272
[3] Liao, J.C., Jay, D.G., etc. (1994) *Proc.Natl.Acad.Sci. USA* 91, 2659-2663

by 25% [4].



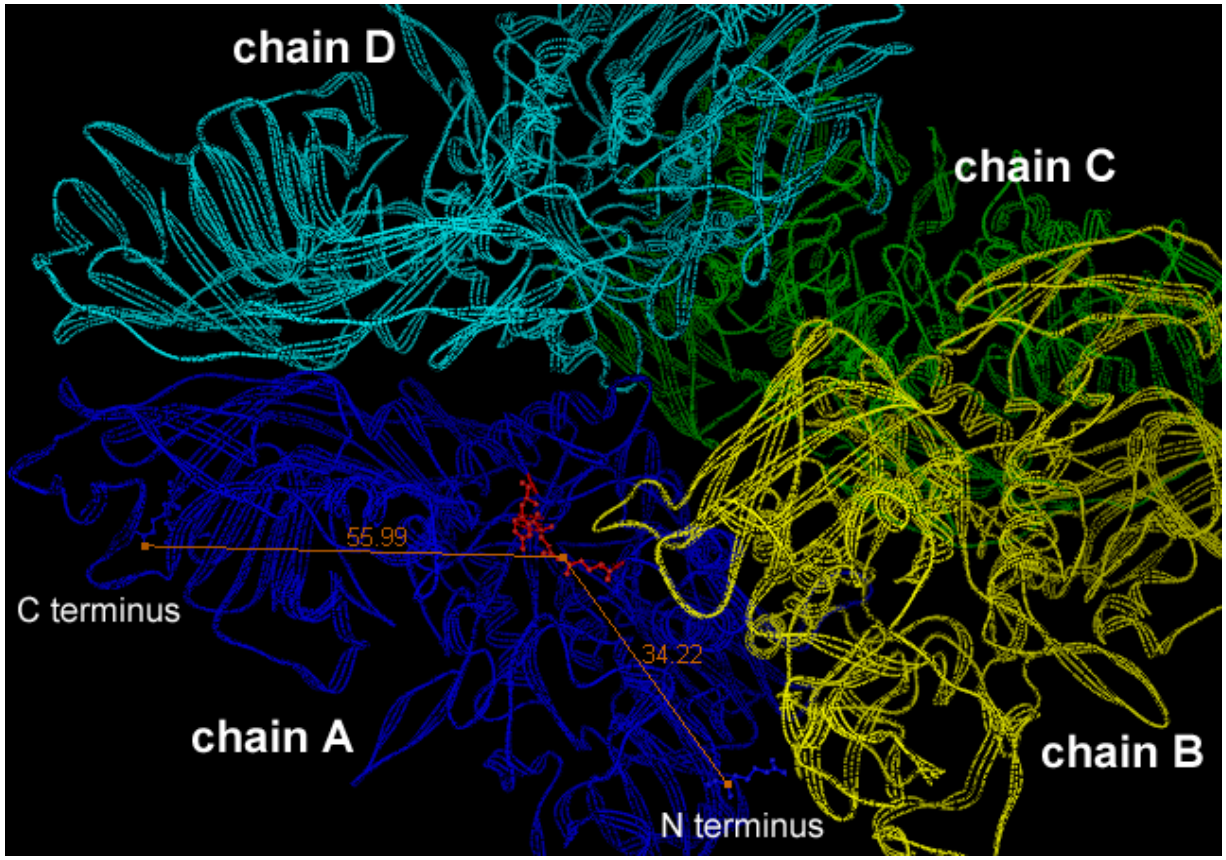
Rajfur *et al* irradiated peripheral focal adhesion of Swiss 3T3 fibroblast expressing EGFP-alpha actinin, and found that stress fibers detached. The retraction of fibers due to EGFP-CALI strongly suggests a functional linkage between alpha actinin and actin-containing stress fibers at focal adhesion [5].



[4] Diefenbach, T.J., Jay, D.G., etc. (2002) *J of Cell Biol*, 158, 1207–1217
[5] Rajfur, Z., Jacobson, K., etc. (2002) *Nature Cell Biol* 4, 286-293

CALI is efficient in cells but mechanism is not well understood.

3. Crystal structure of beta-galactosidase (betaGal)



- Line Ribbon diagram represents the tetramer of betaGal. Different chain is in different color. The active sites of betaGal are shown in red (Glu461, Glu537, Tyr503) [6]. The distances between the active sites to N terminus and C terminus are indicated.
- Greenbaum reported that singlet oxygen generated by GFP photobleaching causes protein damage [7]. Recent studies show that excitation of fluorescein generates singlet oxygen with a half maximal radius of 40 Å [8].

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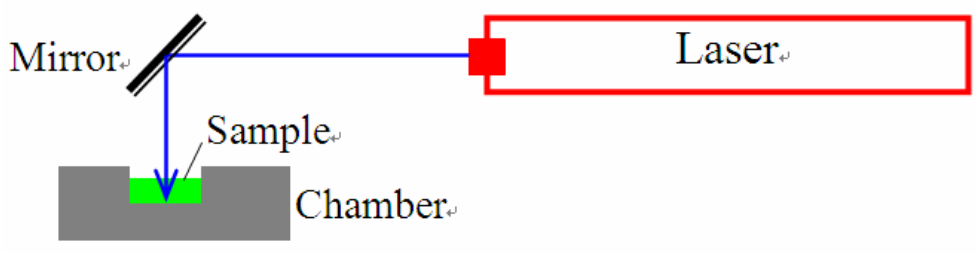
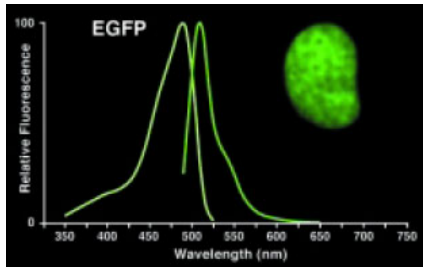
6 Jacobson, R.H., Matthews, B.W., etc. (1994) *Nature* 369, 761-766
7 Greenbaum, L., Malik, Z., etc. (2000) *Bioc chem* 381, 1251-1258
8 Beck, S., Jay, D.G. (2002) *Proteomics* 2, 247-255

METHODS



Most of our *in vitro* CALI was done in this simple system shown in the diagram irradiated with 800 mW for 45 mins. The irradiation source is a Spectra Physics 164 argon ion laser (488 nm line). The activities of betaGal were measured by *High Sensitivity beta-Galactosidase Assay* kit (STRATAGENE®). And the relative activities were calculated by $S = \frac{V_L}{V_{NL}}$ (**V** is the velocity of colorimetric reaction; **L** stands for the sample irradiated by laser, while **NL** stands for the control sample placed in the room temperature). S=1 means that there is no CALI effect.

GFP scanning was used to quantify photobleaching of EGFP tag. According to the emission spectrum of EGFP (*Chroma*®), we use $S = \frac{O.D._{502nm-L}}{O.D._{502nm-NL}}$ to calculate EGFP relative activity.

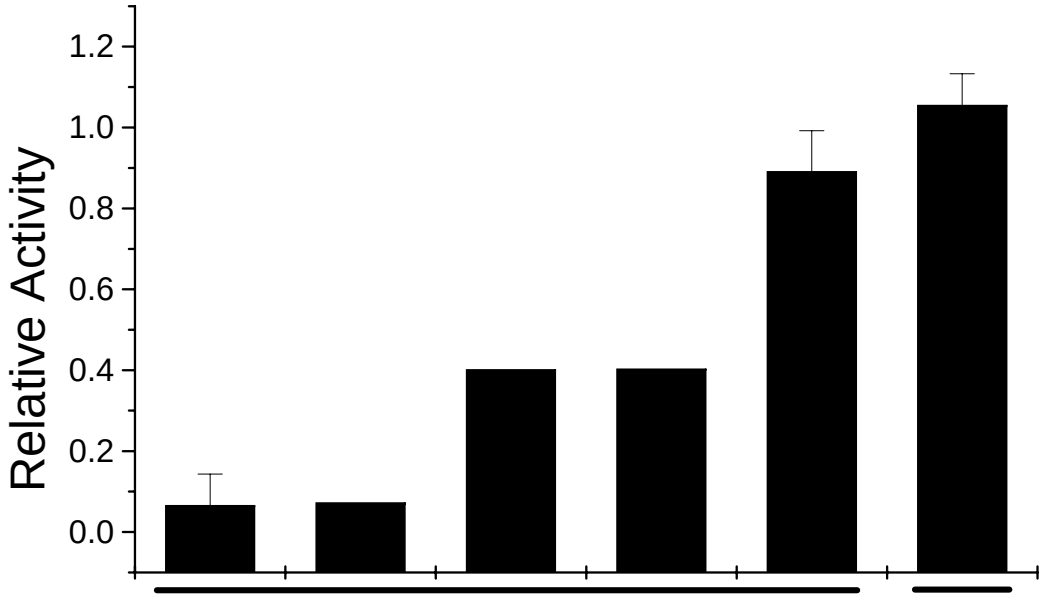


Plasmid constructing, Western blotting and native PAGE were operated following standard protocols. Reagents were purchased from Sigma® and Invitrogen®.

RESULTS

1. Amount of EGFP-CALI depends on irradiation power and duration

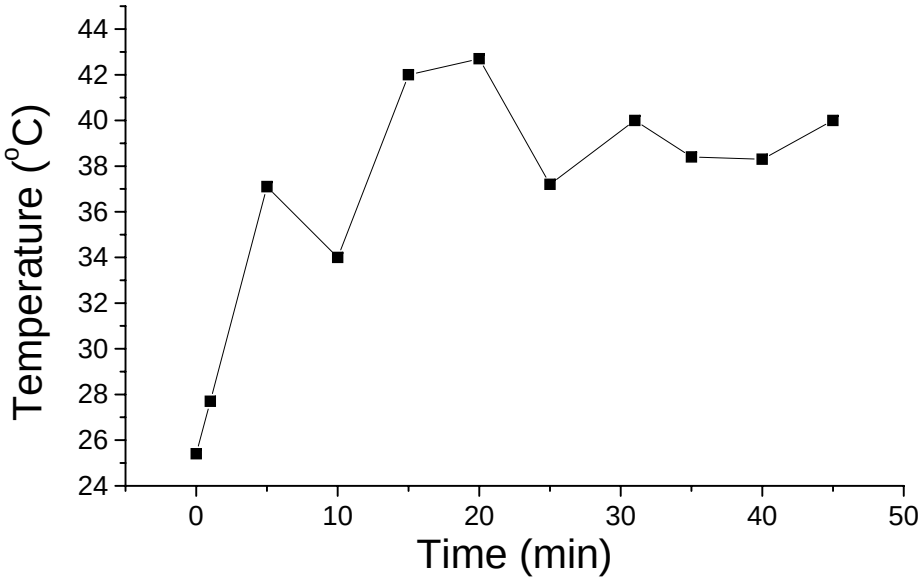
150 ul lysate of EGFP-betaGal was irradiated directly with different power and duration. Then the samples including the control were diluted 6000 fold and analyzed by *High Sensitivity beta-Galactosidase Assay* kit (STRATAGENE®). The $O.D._{590nm}$ readings from the spectrophotometer (UV-1601, Shimadzu®) were used to calculate the betaGal relative activity.

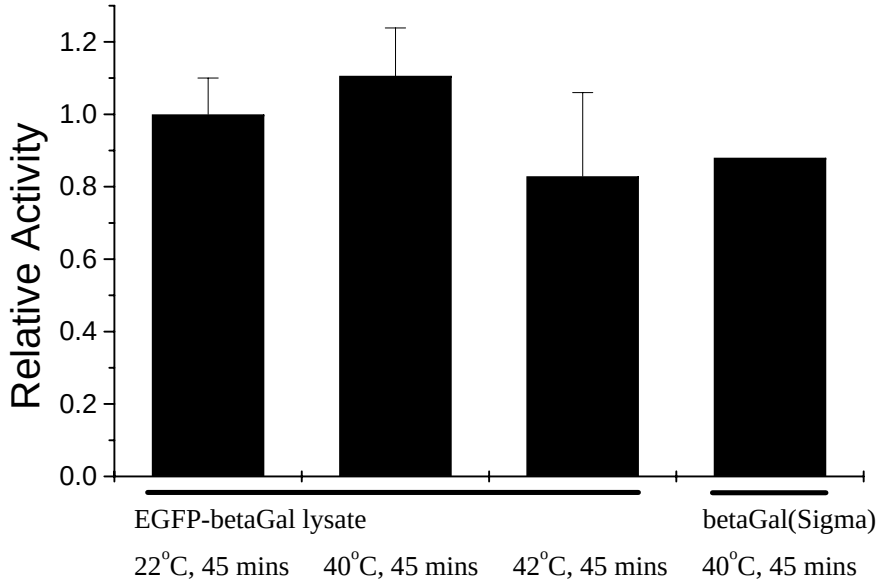


2. Temperature effect on EGFP-CALI is subtle

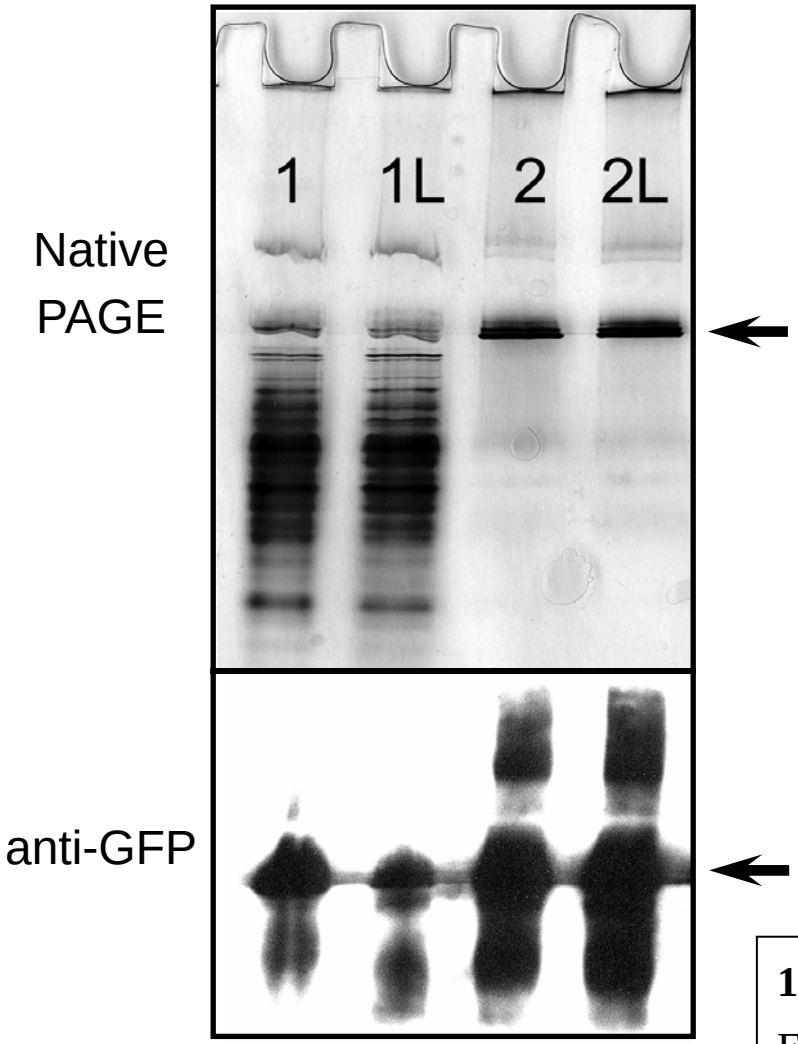
Put temperature sensor into the chamber, and measure the temperature every 5 mins. Results show that the irradiation can heat the sample, from room temperature to around 40 ^{o}C.

In order to exclude the influence from thermal denaturation, we use water bath to incubate samples and measure the relative activities $S = \frac{V_{T(\text{water bath})}}{V_{T(\text{room temperature})}}$ of betaGal. Results show that the temperature effect (activation or inactivation) on the betaGal is about 15%.





3. No protein crosslinking occurs during EGFP-CALI



In order to figure out whether the protein components changes or crosslinking occurs after CALI to influence the enzymatic activity of beta-galactosidase, Native PAGE was employed.

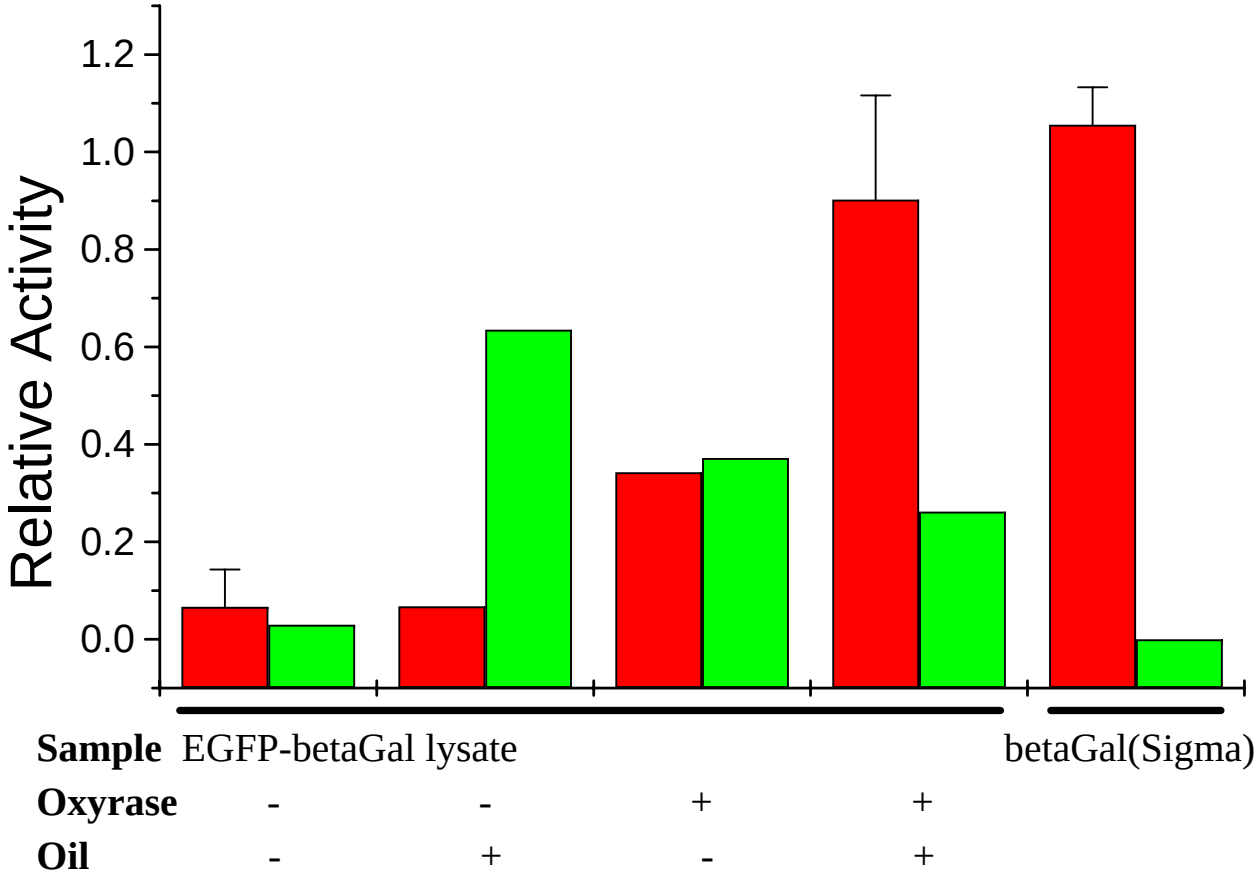
For the native PAGE, the stacking gel's concentration was 4.3%, and the separating gel's concentration was 7%. Coomassive blue staining showed there were no obvious differences between samples with and without irradiation. Western blotting (anti-GFP and anti-betaGal) was used, and again there was no significant difference.

1 is the lysate of EGFP-betaGal, **2** is the purified EGFP-betaGal from 1. **L** means that the sample is irradiated. The arrowheads show the position of EGFP-betaGal.

4. EGFP-CALI requires oxygen

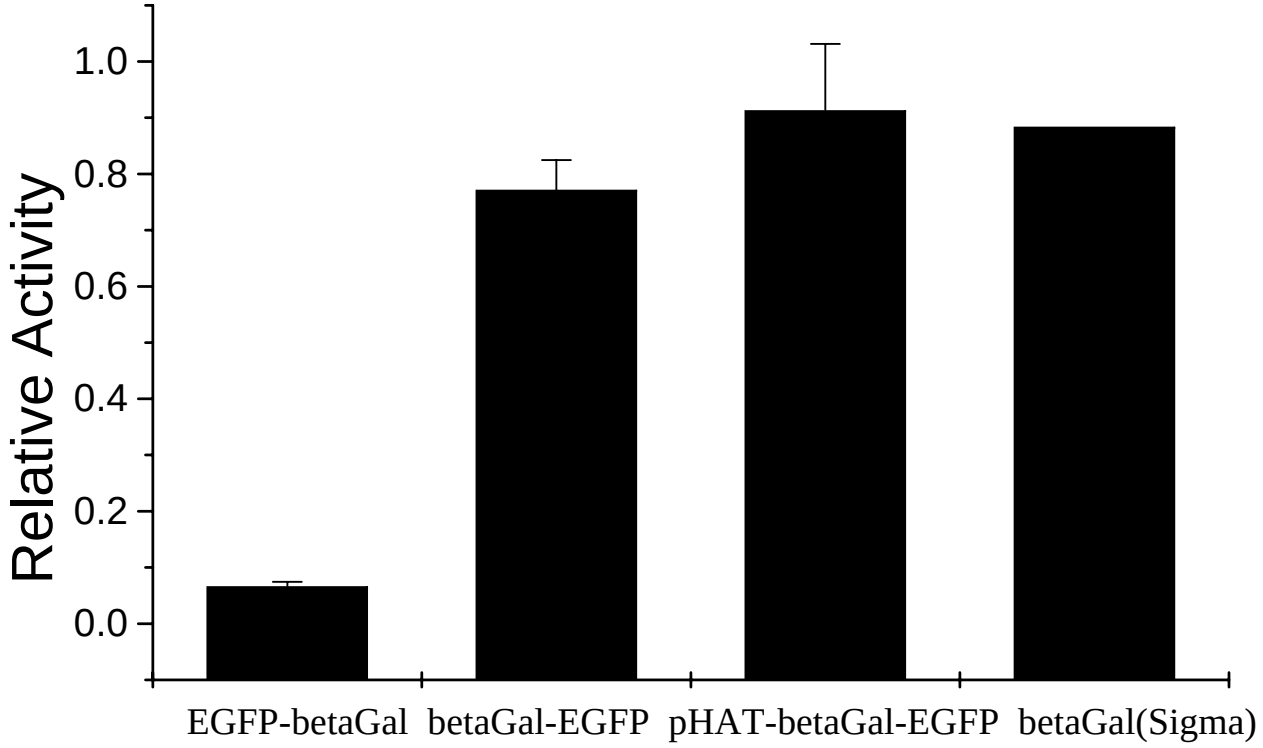
Oxyrase (Oxyrase®) is an enzyme system that naturally, selectively and efficiently removes oxygen from its environment. We mix the sample with oxyrase (3U/ml) to achieve low O_2 condition, cover the mixture with mineral oil (Sigma®) to exclude the molecular oxygen in air, and then do the measurements.

Results show betaGal (red bar) will be protected in the absence of oxygen, while EGFP (green bar) is still photobleached. And mineral oil may decrease the EGFP photobleaching a bit.



5. EGFP position influences CALI results

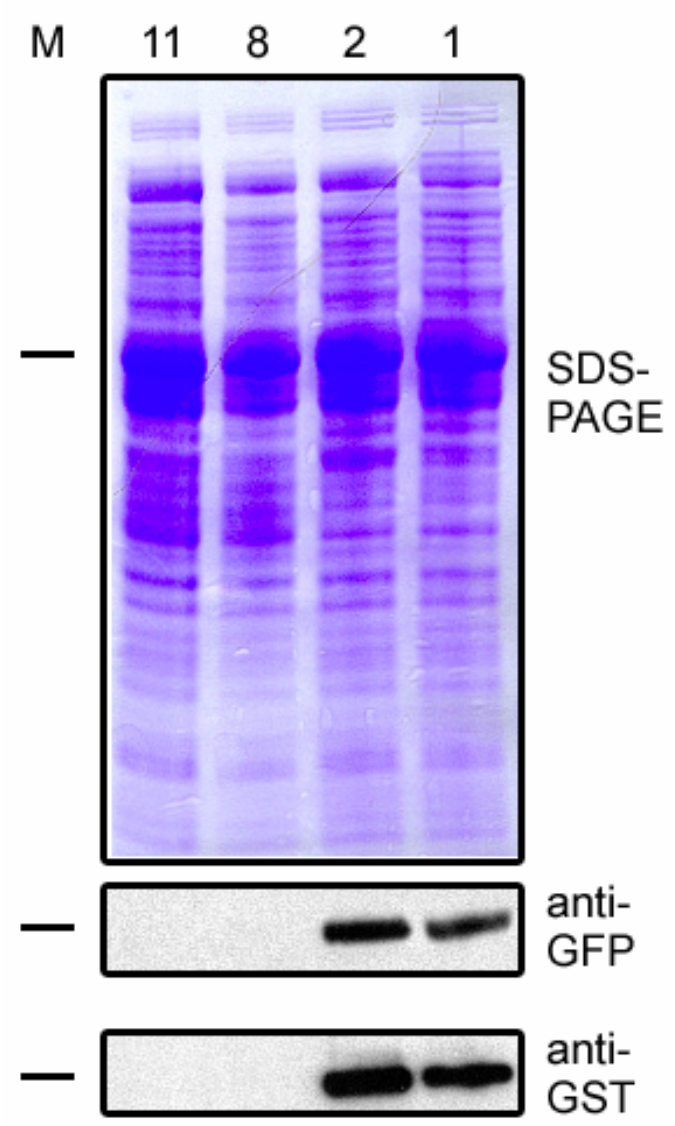
Different constructs were built to express the EGFP tag either at the N terminus or C terminus of betaGal.



The construct pHAT-betaGal-EGFP expresses betaGal fused with hexad-histidine at the N terminus and EGFP tag at the C terminus.

Lysates were made after protein induction and relative activities were measured.

6. New constructs with different target proteins (for future work)



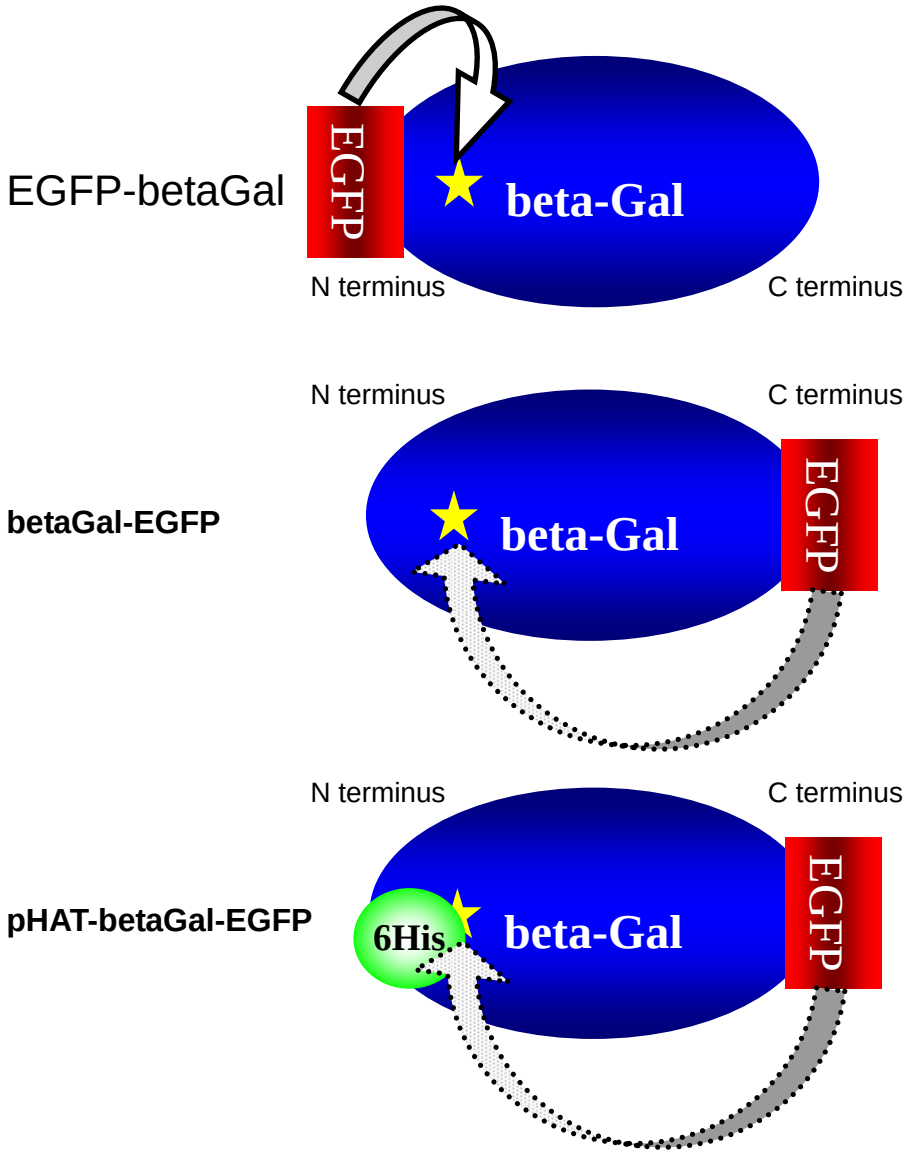
- EGFP-GST series:

EGFP was tagged to either N terminal or C terminal of GST. The figures show that clone 1 and 2 expressed GST-EGFP and can be used in further work (M lane shows the size of the GST-EGFP).

- EGFP-SF9 series:

EGFP was tagged to the N terminal of single chain antibody of Myosin II. This construct will be used in motility assay.

DISCUSSION



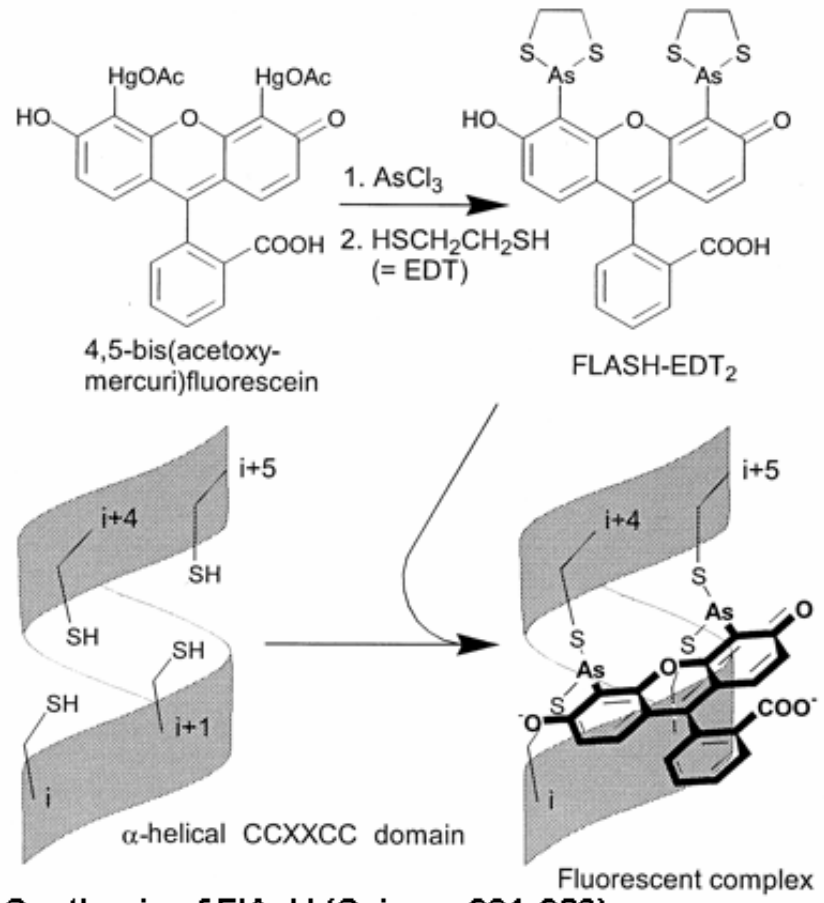
Spatial model of CALI effect

Proposed mechanism: Singlet oxygen generated by irradiation of EGFP attacks beta-galactosidase and alters key amino acids residues near the enzyme active site thereby reducing enzyme activity (half maximal radius of damage around 40 Å). The position of the EGFP relative to the enzyme active site will determine the amount of CALI.

When EGFP is at the N terminus, singlet oxygen will successfully destroy the active site of betaGal (34 Å < 40 Å) and show obvious CALI effect. While

EGFP is at the C terminus, singlet oxygen can't reach the active site (56 Å > 40 Å). And the hexad-histidine-tag may protect the active site more if it caps the active site.

Use of FlAsH (4'5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein) may overcome the spatial problem. This fluorescein derivative [2] recognizes tetracysteine motif in vivo and becomes fluorescent. The fluorophore can be precisely localized by encoding the motif along with DNA for the protein.



Synthesis of FlAsH (*Science* 281:269)

FUTURE WORK

- Quantify the concentrations of EGFP tagged proteins to have a further look at CALI.
- A new chamber was designed to keep the sample's temperature stable during irradiation to minimize the photothermal influence.
- CALI with new EGFP tagged constructs. EGFP-GST series may confirm the results seen with EGFP-betaGal. EGFP-SF9 will be used in *in vitro* motility assay: mixture of polymerized actin and purified EGFP-SF9 protein will be loaded on to the coverslip attached with myosin II; then irradiated and use fluorescent microscopy to see whether the movement of actin filament is inhibited or not.
- FlAsH-CALI.
- Transient transfect EGFP-SF9 into NBT-II cells, and study the proteins interaction or function by CALI.
- Different quenchers to rule out other possible CALI mechanisms.

SUMMARY

- Photothermal denaturation influences EGFP-CALI about 15%.
- EGFP-CALI requires oxygen.
- The degree of EGFP-CALI is related to the distance between EGFP tag and active site.

ACKNOWLEDGEMENT

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