The Study of Dynamic Localized Processes in Cells

The ability to study intracellular processes with high spatial-temporal resolution will lead to a much greater understanding of cellular regulation and communication. High levels of organization exist within cells that are still poorly understood, and the development of methods to probe this intracellular compartmentalization will greatly increase our understanding of cellular mechanisms. At the Shear lab we are developing a method to probe intracellular chemistry in a localized fashion using protein coated submicrometer particles, or beads, and an optical trap. This method has the unique features of being able to probe intracellular chemistry with unprecedented resolution, and of being relatively easily adaptable to the study of numerous enzymes and substrates.

Optical Trapping

Optical Trapping is based upon the principle of conservation of momentum. Light is composed of photons, which have momentum. When light interacts with a substance that causes it absorbs, emit, scatter, reflect, or refract, the direction of its momentum changes. Since momentum is conserved, a force is generated on the interacting object. This force has a direction opposite that of the lights momentum change. With noncoherent light, the size of this force is minimal, a noticeable only to astronomers. With the use of a laser however, it is possible to exert significant forces on small particles. When a laser beam is focused through a high N.A. lens and onto a particle, the forces exerted on the particle are such that the particle moves toward an energy minimum at the laser focal point. Once at the focal point of the laser a particle will tend to remain trapped there, and in fact will move with the laser beam in order to remain at the focal point[1-3].

We are using an inverted microscope as a platform for our optical trap. Light at ~800 nm is produced by continuous wave Titanium: Sapphire laser. This wavelength was chosen due to low absorption by biological molecules in this range. This beam goes through several adjustments before being sent through a high numerical aperture objective in the scope to form a diffraction limited spot. Particles set on the inverted microscope can then be trapped by the beam (Figure 1).

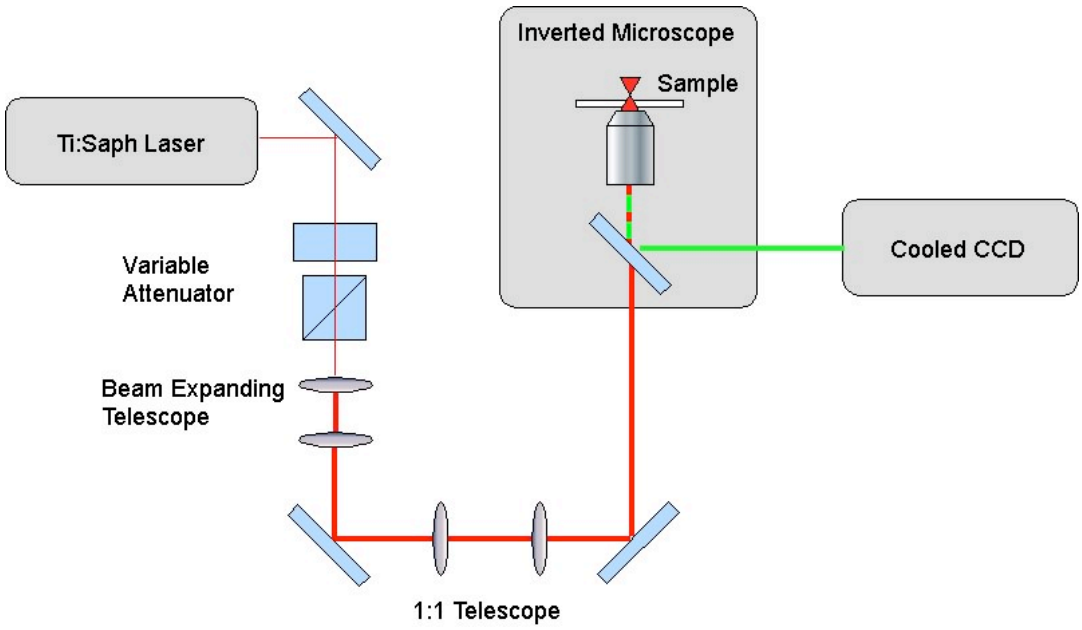

Figure 1. Basic conditions for an optical trap

The optical trap is used to move particles into cells. This can be done by growing cells of interest on slides that are then set on the microscope stage. A solution of beads is added, and a single particle is trapped. By moving the stage with cells on it onto the trapped particle, it is possible to insert the particle into the cell body (Figure 2). The bead solution is prepared in such a fashion that cells are submerged in a 70% hypotonic solution during

loading. This seems to allow for easier loading of the particle into the cell. Once the particle is in the cell, the hypotonic media is removed, and standard media is replaced.

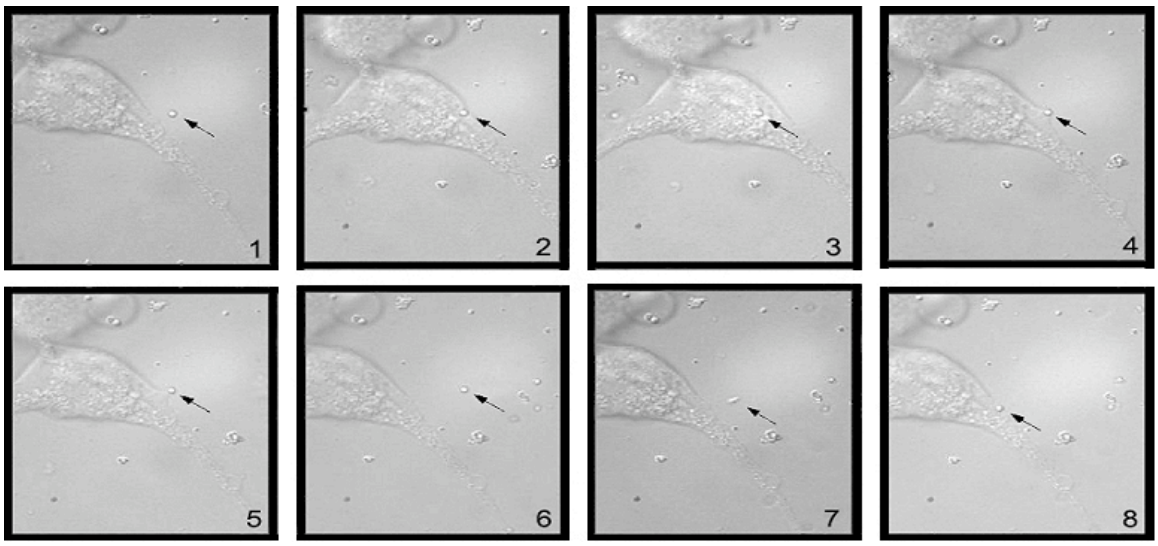

Figure 2. An uncoated 1 μm particle is loaded into an NG-108 cell using an optical trap. The particle remains tethered to the cell when removal is attempted.

The negative effects on the cell of particle insertion seem to be minimal. This was determined using Fluo-3, a molecule that becomes fluorescent upon binding with calcium. Fluo-3 was loaded into cells using pinocytosis before a particle was loaded into the cell. Several hours after loading, the cell showed normal calcium distribution.

Submicrometer Particles

Particles are available in a number of different materials and sizes. Experiments have been performed using latex, agarose, silica, controlled pore glass, and PMMA particles to name a few. Particle sizes for these experiments range from 400 nm to 1 μm. Materials are chosen based on their ability to be trapped, which is generally a function of their refractive index. Large size beads are more easily manipulated but more difficult to

penetrate the cell membrane, while smaller particles penetrate the membrane fairly easily but are problematic to work with. Current experiments use 1μm latex beads (Figure 3).

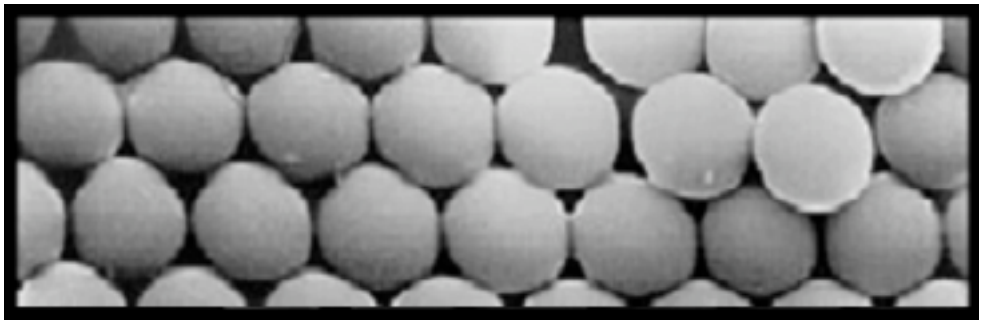

Figure 3. 1 μm diameter latex particles

Coupling Reactions

Particles are commercially available with a variety of surface derivitizations for use in subsequent chemical reactions. Original attempts at coating the particles with protein were focused on using an EDAC reaction (Figure 4). This reaction will couple a carboxylic acid, which the particle is derivitized with, to a primary amine, which are generally available in several places in most proteins. This method for surface derivitization was not pursued due to the necessity for several long incubations. Many enzymes lose activity with excessive time spent at room temperature; so long incubation times often lead to reduced activity.

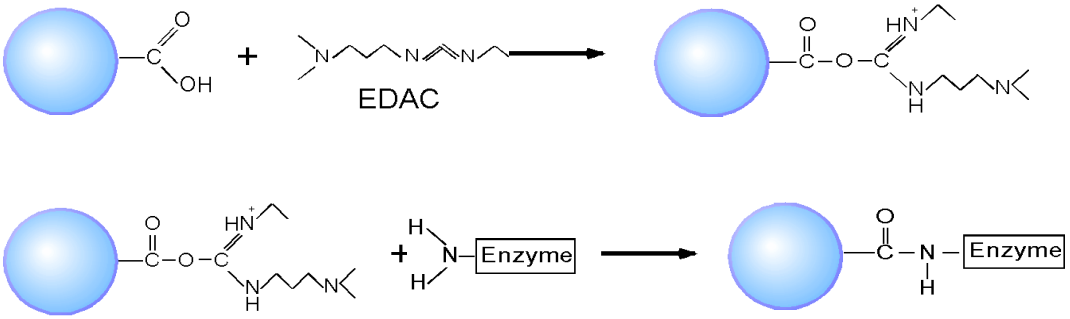

Figure 4. An EDAC reaction couples a carboxylic acid to a primary amine.

Currently, coupling is performed using a biotin avidin binding scheme. 1 μm latex particles are obtained covalently coated with avidin. Each avidin can bind four biotins in one of the strongest noncovalent interactions in nature. The dissociation constant of biotin and avidin is $K = 10^{-15}M$[4]. Biotin is covalently attached to a protein by a succinymidal ester reaction, and the biotin-modified protein is then linked to the avidinated particle (Figure 5).

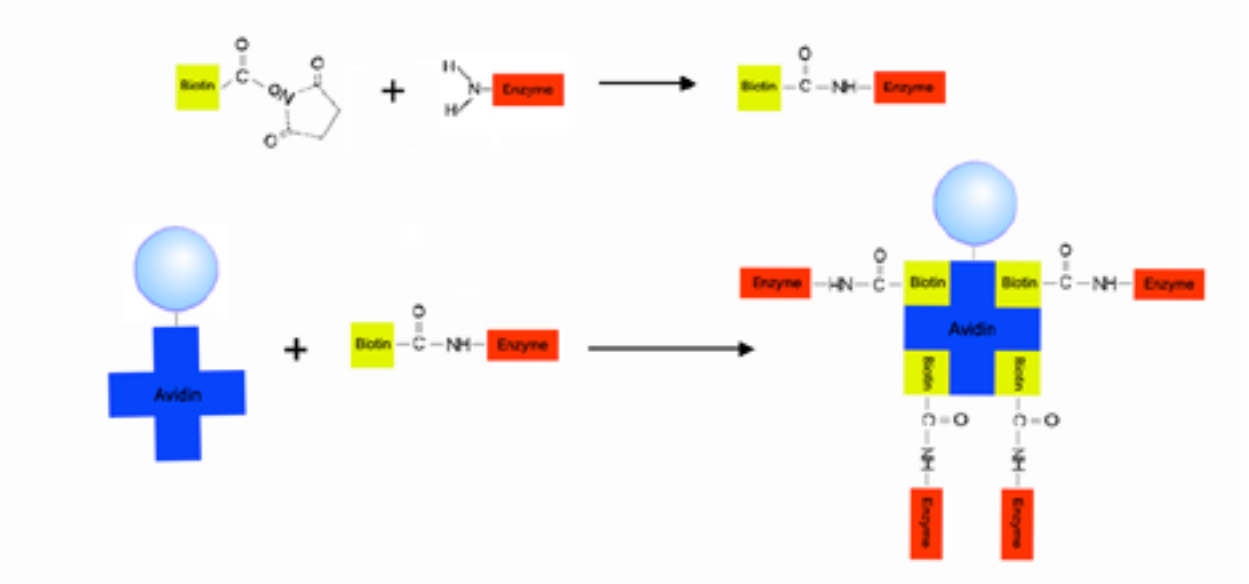

Figure 5. Biotin-Avidin coupling scheme.

The goal of these reactions is to bind with relative ease any protein to a submicrometer particle. Once this has been accomplished, it is possible to insert the protein-coated particle into a cell and study the physiological response.

Proof of Concept

Before a cells reaction to the introduction of a localized signal originating from a particle could be studied, it was necessary to determine if an active particle would retain

its activity upon insertion into a cell. For this purpose, cells were passively loaded with Bodipy-Casein, an intramolecularly quenched protein substrate. This substrate is nonfluorescent until cleaved by the enzyme chymotripsin, at which point the intramolecular quenching is relieved (Figure 6). A particle coated with chymotrypsin relieved this quenching in a localized fashion upon introduction into a cell (Figure 7) indicating that it is possible to perform localized chemistry within a cell by introducing a protein coated particle.

Figure 6. An intramolecularly quenched Bodipy-Casein substrate is cleaved by chymotripsin to form a fluorescent product.

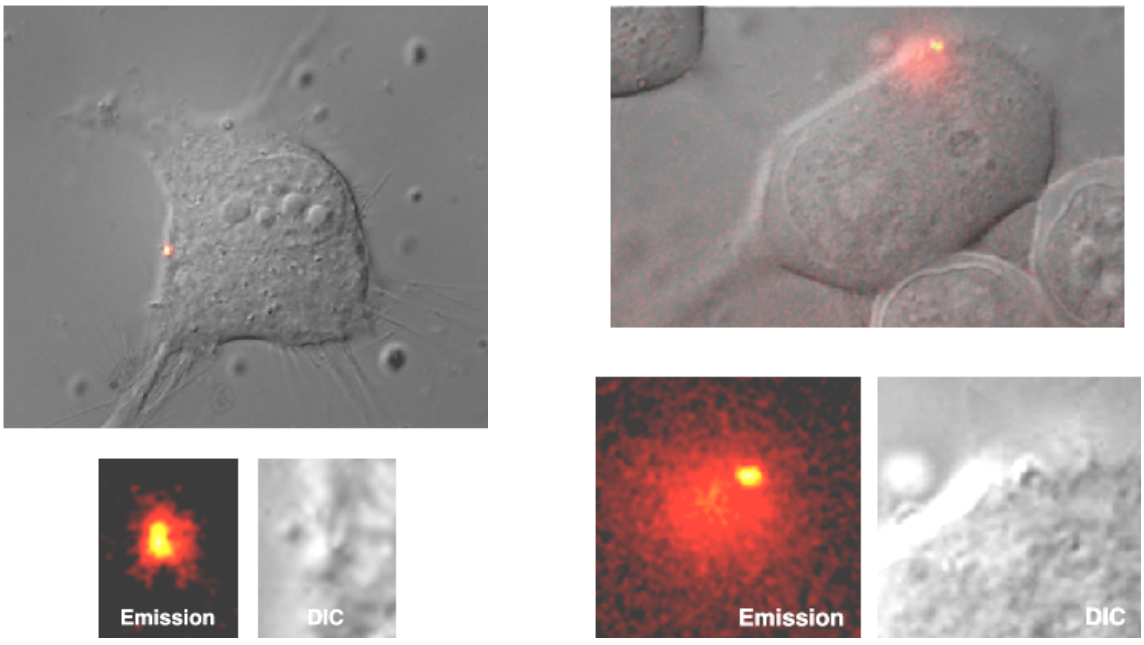

Figure 7. Localized quenching of Bodipy-Casein substrate loaded by pinocytosis is relieved by a chymotripsin-coated particle.

An Enzyme of Interest and its Activity

The first physiologically relevant study performed using the optical trap system was the introduction of a protein kinase A (PKA) coated particle into a cell. This enzyme was chosen due to its major role in many signal transduction cascades including the not yet well understood pathway that leads to neuronal differentiation. The involvement of PKA in differentiation made it an ideal enzyme as its activation in other studies has led to the growth of neurites. The growth of neurites would be an easily detectable result to the introduction of a localized PKA particle, alleviated some of the difficulties inherent in studying enzymes with less obvious responses.

PKA is a tetramer composed of two regulatory subunits, and two catalytic subunits. Regulation of this enzyme is relieved when it interacts with cAMP, at which point the regulatory subunits are released, and the catalytic subunits are free to interact with any one of their numerous substrates. The PKA action is that of a typical kinase, indicating that it phosphorylates its substrate (Figure 8 and 9)[5].

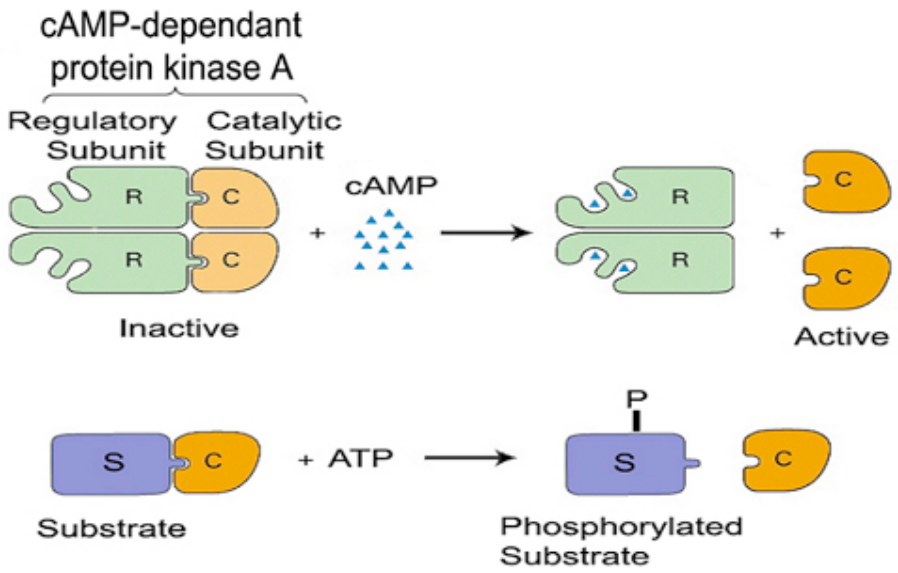

Figure 8.The mechanism of PKA activity.

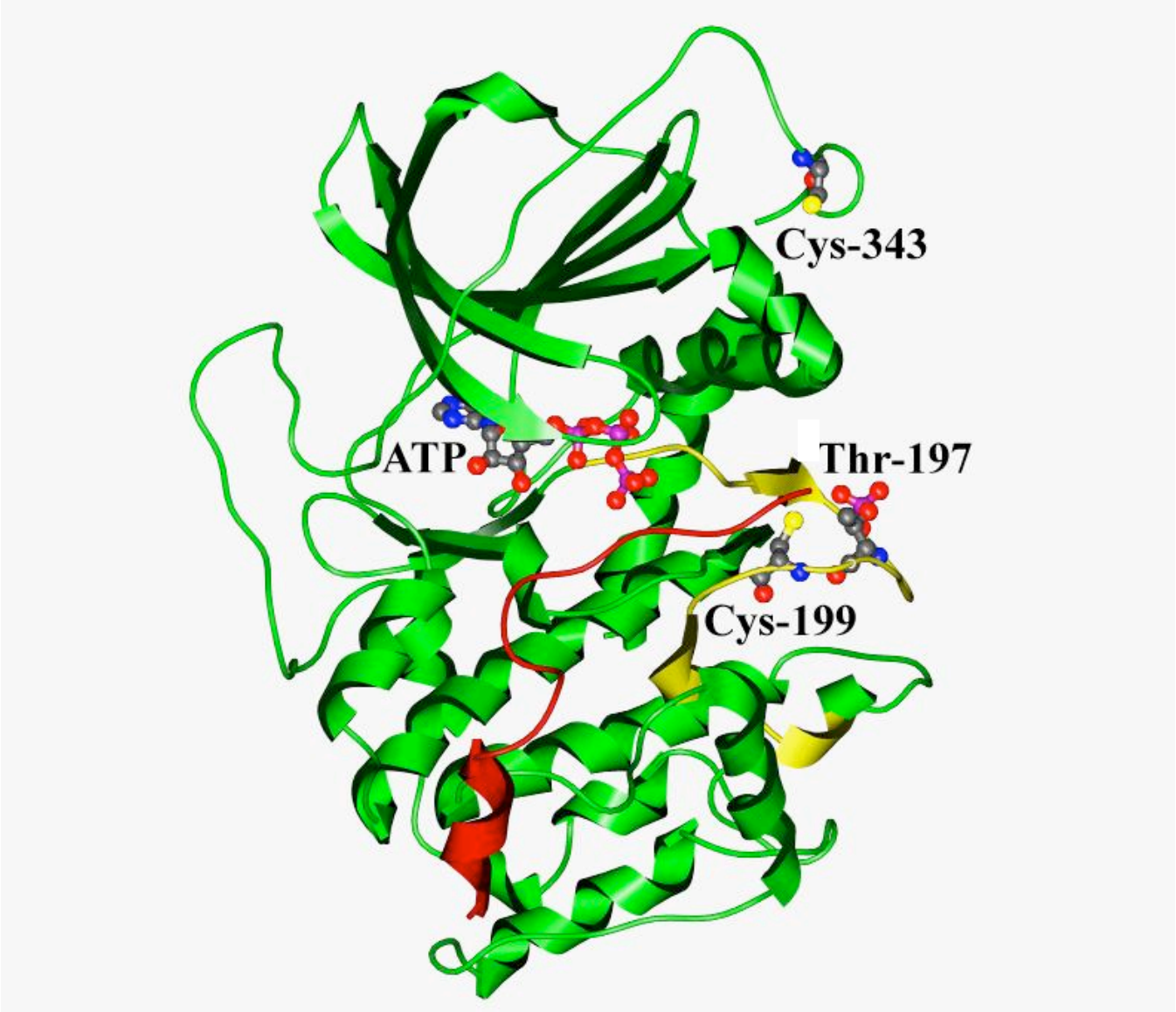

Figure 9. Structure of PKA catalytic subunit

The reaction used to binds a given protein to the particle is not a very specific one. PKA has ~200 primary amines, with some number being free to react with a solution of biotin SE. A single PKA molecule will generally then have several biotins attached to it, all of which are attracted to the avidin on the particles. It is not possible to control which amines the biotin binds to, and it is also not possible to control the position in which a PKA molecule is finally bound to a particle. It is therefore necessary to

determine the PKA activity of a solution of particles before introducing a particle into a cell, as it is possible that a given PKA molecule may have been inactivated either due to the binding of a biotin to a primary amine necessary for enzyme activity, or because the enzyme has been bound to the particle in such a fashion that the active site is not accessible. There is of course no guarantee that activity determinations of the bulk solution will be accurate indications of a single particles activity, but such measurements will be used until it is possible to make accurate activity determinations for single particles.

Detection of PKA activity on particles was first attempted using a Promega Peptag assay kit. This assay provided a fluorescent peptide that is phosphorylated by active PKA. Phosphorylation changes the net charge of the peptide from +1 to –1. This peptide is incubated with the particles in the presence of ATP to provide a phosphate group for transfer. After reaction, the phosphorylated and unphosphorylated peptides are separated by agarose gel electrophoresis (Figure 10). Though this assay works well on standard solutions of PKA, it is not sufficiently sensitive to detect very low levels of the enzyme. Activity of the PKA coated particles was not detected, and it was assumed that the levels phosphorylated peptide generated by the particles were too low to be detectable by this method.

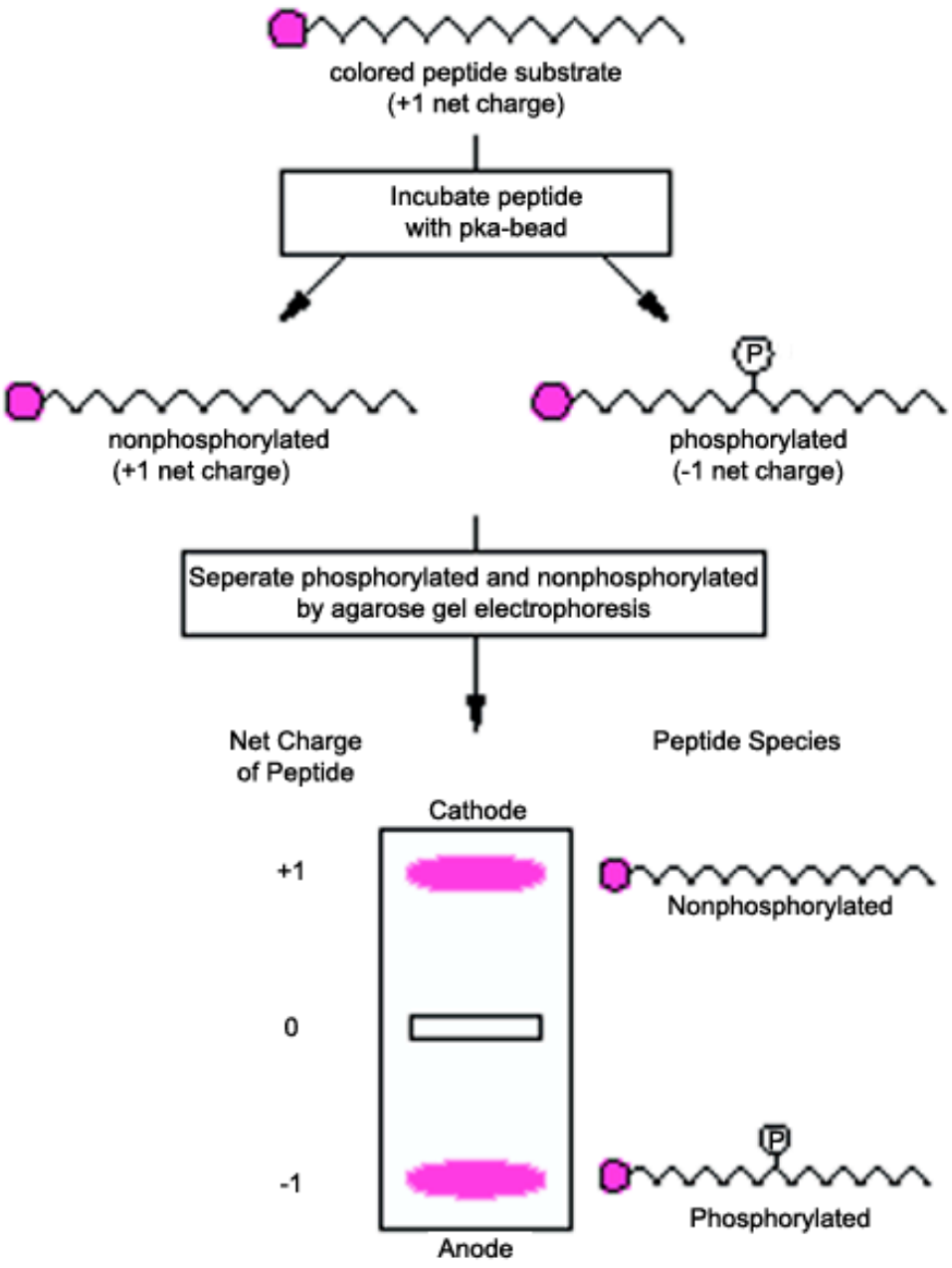

Figure 10. Mechanism of PKA activity detection using a Promega Peptag PKA assay kit

A Promega Profluor assay was subsequently used to detect PKA activity on the particles. This assay consists of an intramolecularly quenched fluorescent peptide substrate for PKA. Upon cleavage by a protease, the peptide becomes fluorescent. Phosphorylation by PKA prevents the necessary cleavage by the protease, making

phosphorylated peptides nonfluorescent. Increasing PKA activity is detected by decreased of fluorescence (Figure 11).

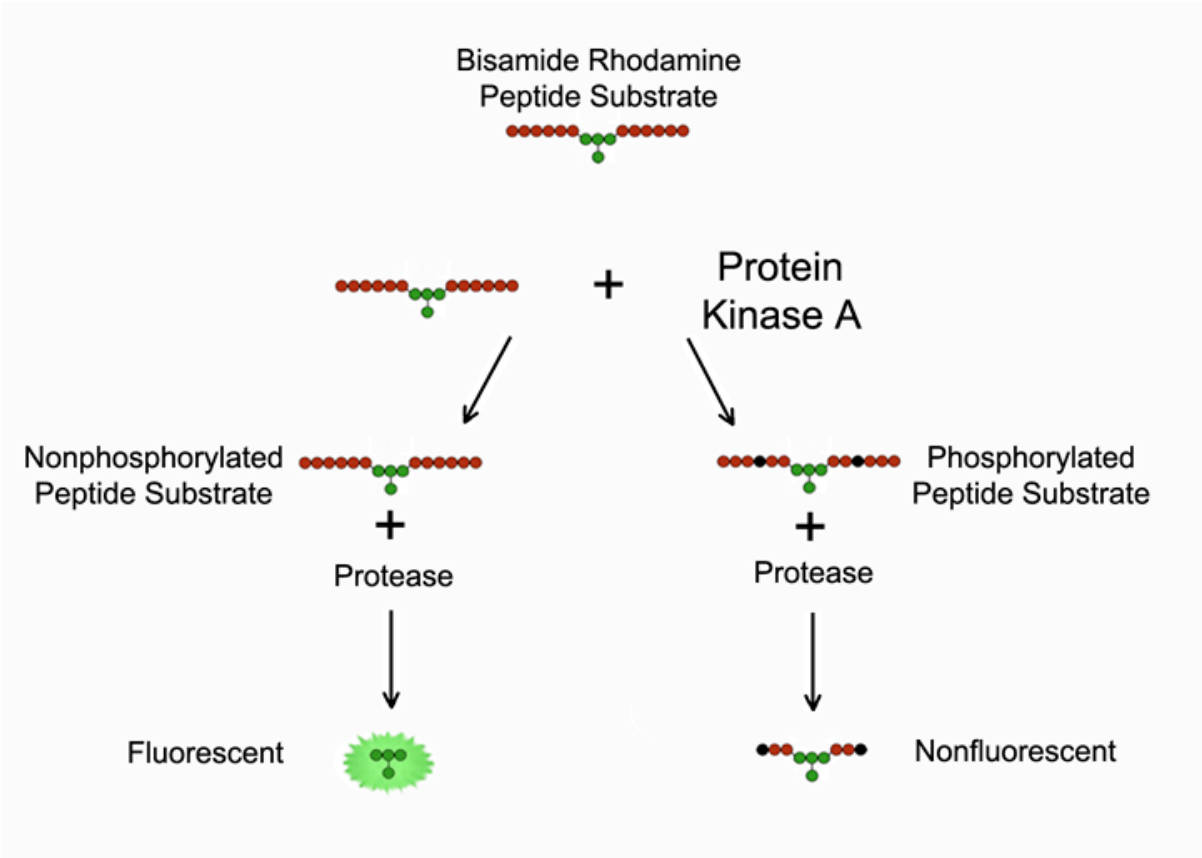

Figure 11. Mechanism of PKA activity detection using a Promega Profluor PKA assay kit

Quantification of PKA activity is accomplished by fluorescence measurements on a plate reader. A standard curve of PKA activity is prepared using solution phase PKA of known activity. Fluorescence from the PKA coated particles is compared to these values in order to quantify the unknown PKA activity. The presence of the particles in solution seems to inhibit the action of the assay to some degree. In order to account for this interference, the fluorescence from PKA particles is compared to the fluorescence from

PKA particles to which no ATP is added. In this manner, it is possible to compare the fluorescence of the PKA particles to identical particles expressing no PKA activity, thereby accounting for whatever fluorescence inhibition the presence of the particles causes. The difference between the fluorescence values of the active and nonactive PKA particles is then considered to be the value attributable to the actual PKA activity on the particle.

Current work on this assay is focused on increasing the number of points on the standard curve in order to determine the linear range. It appears that values are not currently available for the linear portion of the curve, limiting its usefulness for activity calculations. The difference between a set of activated and nonactivated PKA particles has been determined, and it can be said with 99% certainty using a students t test that the values are distinct, indicating that there is measurable PKA activity on the particles [6].

Experimental

The insertion of PKA coated particles into cells produced some interesting results on a number of occasions. Using NG-108 cells, a neuroblastoma glioma cell fusion line, a neurite was observed to grow in the location the PKA particle over a four hour period on one occasion (Figure 12). This result has not yet been reproduced, but numerous control studies with both protein-coated and non protein-coated particles have never produced this result.

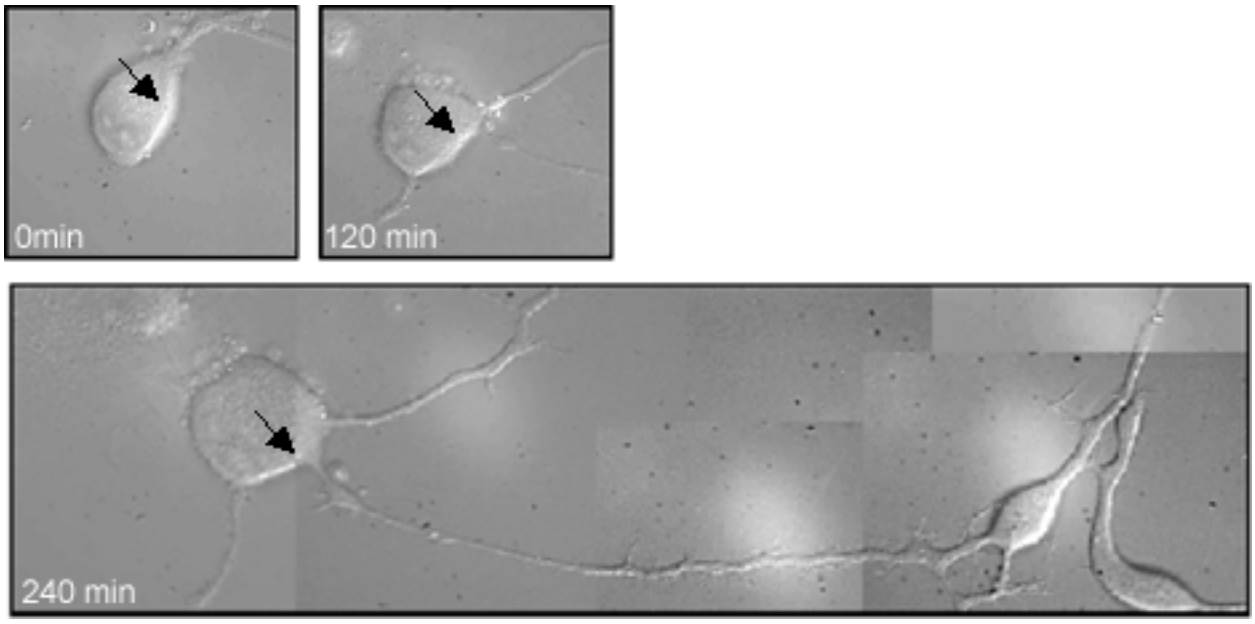

Figure 12. A PKA coated particle is introduced into an NG-108 cell. Over a four-hour period neurite has grown at the location of the particle.

Introduction of PKA coated particles into primary culture ventral striatum cells from three day old postnatal rats produced somewhat different but also interesting results. Introducing a PKA coated particle into a cell with an existing neurite in the vicinity caused a colocalization of neurite and particle (Figure 13). The mechanism of this action is not yet understood, but it has been observed with some regularity. It is not clear how much of the colocalization is due to movement of the cell, and how much to movement the particle. Future studies will use a normal trapping beam to move the particle into the cell, and then reduce the power of the beam while maintaining the particle in one position rather then releasing it and allowing it to move about the cell. By lowering the power of the beam damage to the cell will be minimized while still maintaining control of the particle.

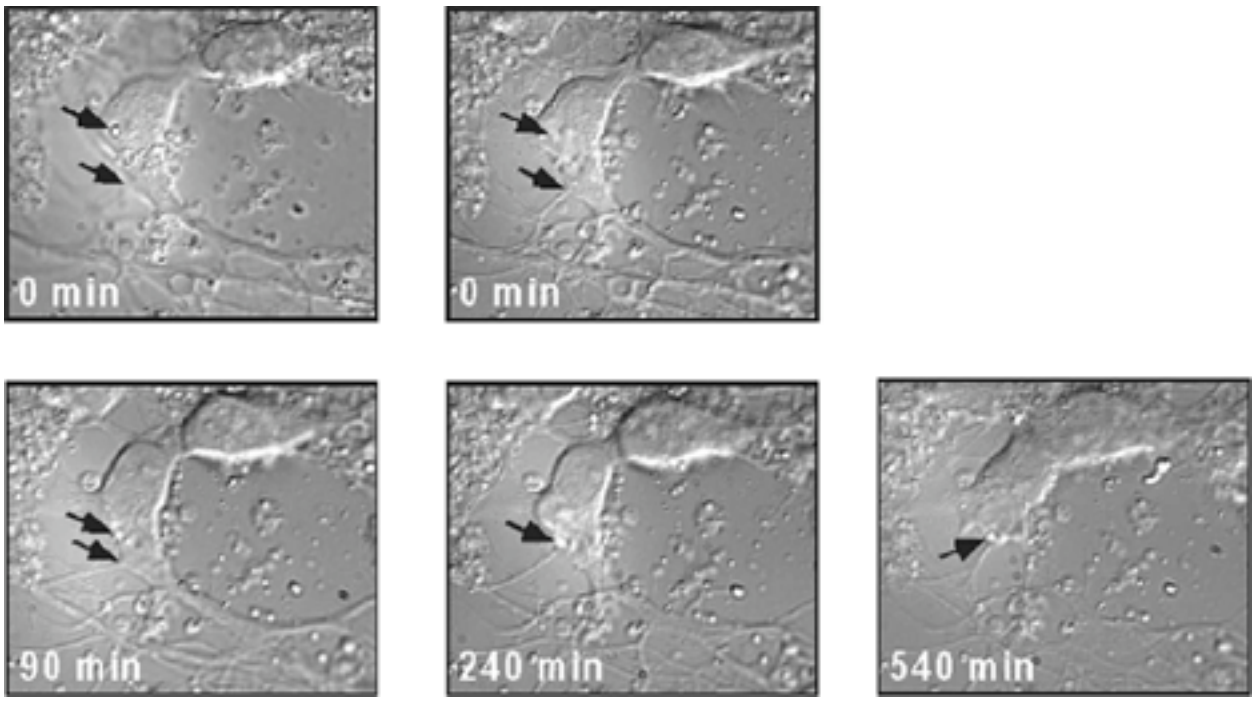

Figure 13. A PKA coated particle is introduced into a ventral striatum cell cultured from a three day old post-natal rat. Over a four-hour period a preexisting neurite has colocalized with the particle.

Results and observations stemming from studies of PKA coated particle introduction into cells are quite promising. Future work will focus on several areas of the experimental setup. First, it is necessary that the reliability of the cell loading procedure be increased. It is not currently possible to choose a specific location within a given cell and reliably place a particle there. This ability is necessary if this technique is to provide valuable information about localized signals in cells. Also, increased viability of cells upon the stage is of utmost importance. It is possible to study cellular reactions only as long as the cell is alive and healthy on the stage. Extending this time is crucial to further studies.

Future work with this technique will focus on several areas. Cellular reactions to the introduction of particles coated with other enzymes and substrates will be studies. Currently, other members of the Shear lab are working to introduce RNA coated particles into the membrane to study localized protein synthesis. Also, methods are being developed to remove particles from the cell after an incubation time, and analyze them by other analytical means. This could provide information about enzyme activities on particle bound substrates within a cell, and essentially allow for the study of a step of a signal transduction cascade in a single location.

References

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