Single cell physiology

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Abstract The possibility to control at specific times and specific places the activity of biomolecules (enzymes, transcription factors, RNA, hormones, etc.) is opening up new opportunities in the study of physiological processes at the single cell level in a live organism. Most existing gene expression systems allow for tissue specific induction upon feeding the organism with exogenous inducers (e.g. tetracycline). Local genetic control has earlier been achieved by micro-injection of the relevant inducer/repressor molecule but this is an invasive and possibly traumatic technique. Here we will present the requirements for a non-invasive optical control of the activity of biomolecules and review the recent advances in this new field of research.

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Introduction

Living organisms are made of cells that are capable of responding to external signals (food, hormones, neurotransmitters, morphogens, etc.) by modifying their internal state (e.g. gene expression or protein phosphorylation patterns) and subsequently their external environment (ionic concentration, pH, release of signaling molecules or enzymes). Revealing and understanding the spatio-temporal dynamics of these complex interaction networks is the subject of a field known as systems biology [1, 2]. In multicellular organisms in particular, cellular differentiation and intracellular signaling are essential for the coordinated development and behavior of the organism. While many of the actors that play a major role in these processes are known (for example morphogens in development and a variety of kinases in signal transduction), much less is known of the quantitative rules that govern their interaction with one another and with other cellular players (such as the type of complexes, rate constants, diffusion range, strength of feedback or feedforward loops). To investigate these interactions (a necessary step before understanding or modeling them) one needs to develop means to control or interfere spatially and temporally with these processes.

One of the crudest and oldest means to interfere with developmental networks has been to use mutants (or to create conditional mutants) for one of the molecular actors and look for the resulting response of the organism e.g. the phenotype. While this approach has been very successful in identifying the qualitative features, such as the other actors and topology of a network (for example the genes downstream of a given regulation factor), it cannot yield quantitative information on the network i.e. the affinities, rate and diffusion constants or type of nonlinearities.

Moreover individual genes may be expressed in a temporally, spatially and tissue specific manner and may be involved in different biological networks at different times. To study those systems, tools have been developed to control gene expression at different stages of development [3]. Systems have been engineered to induce or excise genes [4]; these constructs allow gene expression patterns to be temporally controlled. However the quick (minutes) and fine spatial (cellular) control over protein activity or gene expression patterns is still problematic. In particular, it is impossible to analyze *in vivo* the specific influence of a given cell on a whole tissue or study its response to controlled alterations of the behavior of neighboring cells, phenomena that are anticipated to be extremely important in embryogenesis, organ regeneration and cancer biology. Thus, if one could quickly and locally release or activate a given morphogen, one could perturb the associated developmental network and learn about its spatio-temporal dynamics. Similarly if one could irreversibly label a given cell in a tissue, one might monitor its progeny and thus identify the stem cells implicated in tissue regeneration or investigate the growth of a tumor from a single cell.

To address biological processes at the single cell level in a live organism, electroporation techniques have been developed [5]. They involve electroporation by injection of various molecules (e.g. DNA, RNA or morpholinos) present in a micropipette brought in the close vicinity of the targeted cell, see Fig.1. The technique

is invasive and the amount of electroporated material is unknown. Moreover, because the whole tissue is displaced by the inserted micropipette, the success rate in targeting a specific cell in a live embryo is very low. It would be desirable if a non-invasive technique could be devised to control the rapid activation of a known concentration of biomolecules in a specific cell of a live organism.

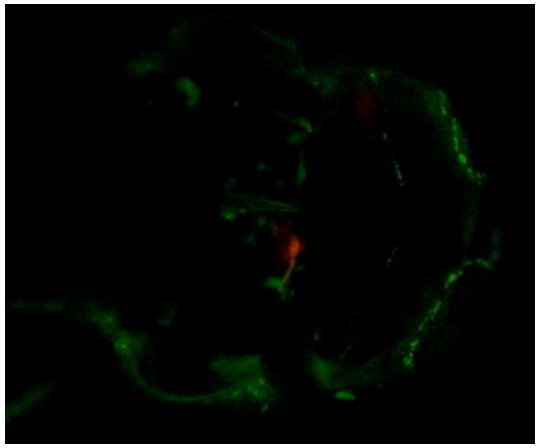

Fig. 1 Electroporation of red-fluorescently labelled LNAs in a single Mauthner neuron of a live zebrafish embryo. The reticulospinal neurons were labeled by retrograde transport of green-fluorescently labeled dextran injected in the tail of a 5 day old fish.

In fact such a technique exists. It implies the photoactivation of caged molecules. Various biomolecules (neurotransmitters, hormones, RNA, proteins, etc.) can be inactivated (caged) by their covalent binding to appropriate chemical groups [6, 7, 8]. Upon illumination, the bond between the biomolecule and the caging group is broken and the molecule thus activated. To locally activate a caged molecule that has reached its target, two-photon excitation is the method of choice [9]: being a non-linear phenomenon, it is limited to the focal point of the illuminating laser beam and molecules along the optical path are not affected. Uncaging, which implies the breaking of covalent bonds, is an energetically demanding process that usually requires UV (365 nm) light to proceed. With two-photon processes, that requirement translates into near infrared (IR) pulsed laser beams (e.g. Ti-sapphire laser emission at 730 nm). These IR pulses have the added advantage over visible and UV light to be less scattered and to penetrate deeper into the tissues. To be non-detrimental to the illuminated cells, the IR beam should however be of the smallest possible intensity (a few mW, or less than 1 MW/cm^2 at the focal point [10, 11]). This sets a not too severe constraint on the two-photon absorption cross section of the caging groups to be used. Finally, as far as the caging design is concerned, it will be very useful to quantify the concentration of molecules photoactivated in a single cell. To that purpose the development of efficient caging groups that become fluorescent upon uncaging has been undertaken [12].

Among the various molecules that have been caged, one can differentiate between endogenous factors (present in the organism) and exogenous molecules (absent from it). The photo-activation of endogenous molecules such as hormones,

retinoic acid, or neurotransmitters allows one to interfere with and learn about the cellular networks of the organism where these molecules play a role. The activation of exogenous factors allows to control the expression of genes introduced in specific transgenic animals.

The existence of caged molecules is a necessary but insufficient condition to control non-invasively processes at the single cell level in a live organism. To achieve that goal, the caged molecules have to reach their target cell(s) and therefore be sufficiently small and soluble to pass through the various physiological obstacles on their way (chorion, epithelium, cell membrane, etc.). This imposes severe restrictions on the type of molecules and caging groups that can be photo-activated in a live organism. Proteins and polynucleotides for example are too bulky. To reach every cell in an embryo, they have to be injected at the single cell stage. Their dilution and eventual degradation limit their usefulness to early stages in embryogenesis. As we shall see below, we have identified retinoic acid (a morphogen) and steroid-like hormones (in particular the non-endogenous cyclofen and its analogues) as good candidate molecules for caging, capable of reaching all cells in a live organism.

1 Caged molecules

The caging of small molecules such as Ca^{2+}, cATP, cGTP, glutamate or serotonin has been achieved a long time ago: the first molecule to be caged and used in a cell context was cAMP[13] in 1977, see also some recent reviews [6, 7, 8]. When planning to cage biomolecules, issues of solubility, stability, cellular toxicity, uncaging cross-section and uncaging kinetics have to be taken into consideration. Many photolabile protecting groups are presently available for caging various chemical functionalities. Despite such a large collection, caged molecules still experience restrictions to face biological situations, in particular in live organisms. In that respect, the main current development in the field of caging groups deals with improving their photophysical and photochemical properties (in particular red-shift of absorption with one-photon excitation or increase of the uncaging cross sections with one- and two-photon excitation). Another current trend concerns the reversibility of photoactivation. This feature cannot be reached with conventional caging moieties as kinetics and selectivity are not favorable to link again two fragments which originate from light-induced bond breaking. In contrast, photochromes which may exhibit major conformational changes upon illuminating can be used to design molecules switching between several states of different biological activities [6, 7, 8].

Caged Ca^{2+} [14] has been the most widely used caged compound. This fact can be explained by the importance of Ca^{2+} in cellular physiology and the existence of Ca^{2+} sensitive dyes. It has led to tremendous progress in the understanding of neurotransmitter secretion.

Caged ATP is another caged compound which led to great insights in enzymatic reactions in the past decades. Na^+-K^+ ATPase properties have been extensively studied over the years, first to determine the characteristics of Na^+ flow [15] then the

pump current properties [16]. Other significant results include the study of molecular motors among which muscle fibers relaxation kinetics [17], myosin orientation [18] and ultimately of force generation characteristics of kinesin at the single molecule level [19].

Caged cAMP has been particularly useful in neuroscience to decouple the neuron response to the natural neuronal stimulation by bypassing the activation of the pathway especially for olfactory transduction [20, 21] or for axon guidance during the establishment of a neural map [22].

Apart from those compounds, only proof of principles have been published for most of the other caged molecules.

2 Optical control in Neurophysiology

In one area, neurophysiology, much progress has been made in the study of the fast spatio-temporal dynamics of neuronal networks by using photoactivation methods to trigger the response of a single cell to an influx of calcium (using photoactivable chelators of Ca^{2+}) and neurotransmitters (using for example caged analogs of glutamate or serotonin) or more recently by controlling the opening/closing of light-sensitive transmembrane channels in cell cultures [23] and in live organisms [24].

Glutamate is a neurotransmitter of choice as it can stimulate almost all the different kinds of vertebrate neurons. Glutamate uncaging can mimic synaptic input, lead to the generation of action potentials, and has been used to map glutamate sensitivity or neuronal connections. Its use with two-photon excitation has led to maps of exquisite resolution [25] and has allowed the study of long term potentiation at the single spine level in a structural and mechanistic way [26, 27, 28].

More recently light-sensitive ionic channels and pumps have been engineered [29]. Two approaches have been taken: one involves the binding of photo-sensitive chemical groups to the channels allowing their activation/inactivation upon illumination, whereas the second directly use the intrinsic light-sensitivity of natural channels. The first strategy [30] uses the vast conformational changes upon photoisomerization of molecules such as azobenzene: UV can trigger trans to cis isomerization, cis to trans being triggered by a longer wavelength or by thermal energy. Such a molecule can be attached to the channel ligand (and thus hide or reveal the ligand) or crosslinked to the actual protein (and thus induce conformational changes in the protein upon illumination). Light is then used at will to turn on or off the channel. The second strategy uses light-activated opsin-based channels or pumps [31]: channelrhodopsin 2 (ChR2) (or halorhodopsin (NpHR)) to depolarize (or hyperpolarize) the cell upon illumination with light at 450 nm (or 560 nm), see Fig.2. This approach is particularly promising since the channels can be expressed in transgenic animals where they can be excited with light at the appropriate wavelength. Using two-photon excitation, it might even be possible to target specific synapses and investigate the neural response of a live animal to such very local non-invasive excitations. The coupling of these optical techniques with the existing Ca^{2+} sensitive dyes

will allow the *in vivo* study of neural networks with unprecedented spatio-temporal resolution [32]. This technique is bound to revolutionize neurophysiology the way patch-clamp did thirty years ago. Indeed, it has already been shown in a live mouse that, upon expression of channelrhodopsin in its brain, neurons can generate spike trains which frequency similar to the one of the excitatory light source. Moreover, the mouse can be trained to respond to a light stimulation [33]. This study shows that such a technology can be readily implemented in a freely moving mouse.

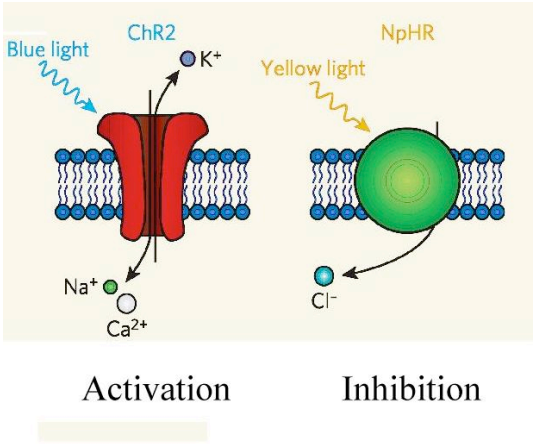

Fig. 2 Depolarization (activation) and hyperpolarization (inhibition) of a neuron by the rhodopsin-based light-activated channels ChR2 and the chloride pump NpHR. Reprinted with permission from Häusser and Smith [29].

3 Optical control of gene expression

The standard way to gain control of gene expression in a live organism is through the use of inducible tissue specific promoters. In appropriate transgenic animals, a desirable gene (for example GFP) is put under the control of a tissue specific promoter responsive to an exogenous inducer (an antibiotic such as tetracycline or doxycycline [34] or a hormone such as ecdysone [35]) whose receptor has also been engineered in the animal. By controlling the diet of the animal (feeding it or not with the inducer molecule) the responsive gene can be turned on or off. Various inducers have been caged e.g. ecdysone [36], doxycyclin [37], and their effect upon photo-activation in cell cultures has been demonstrated, typically by turning on the expression of GFP or $\beta-$galactosidase. So far their photo-activation in a live organism has not been demonstrated, although it should in principle be possible using two-photon illumination to release the caged inducer in a single cell of the organism. Other considerations, mainly toxicity and permeability, may so far have hindered their use in a live organism.

4 Optical control of RNA expression

The optical control of RNA expression has been reported using an approach known as "statistical backbone caging" of polynucleotides [38]. In this approach, a number of sites (about 30) on a mRNA (about 1kb long) are blocked with a photo-cleavable coumarin moiety. The mRNA was injected in a live zebrafish embryo at the single cell stage. The blocked mRNA was transcriptionally inactive, but could be activated when illuminated with UV light (at 365 nm). If the RNA coded for GFP, some of the illuminated embryos would turn fluorescent whereas if it coded for the transcription factor engrailed, some of the embryos would show developmental abnormalities (small or no eyes) after UV illumination [38], see Fig.3. However, due to the statistical nature of the caging/uncaging reactions, this approach yields results with great variability and low reproducibility. More recent work have described a method to overcome this problem by synthesizing a polynucleotide chain (e.g. a morpholino) tethered to a short complementary oligomer with a photo-cleavable linker. When injected at the one-cell stage in a zebrafish embryo, the morpholino could be released at a later stage by UV illumination. The embryo then exhibited the same phenotype as mutants lacking the gene targeted by the morpholino [39, 40].

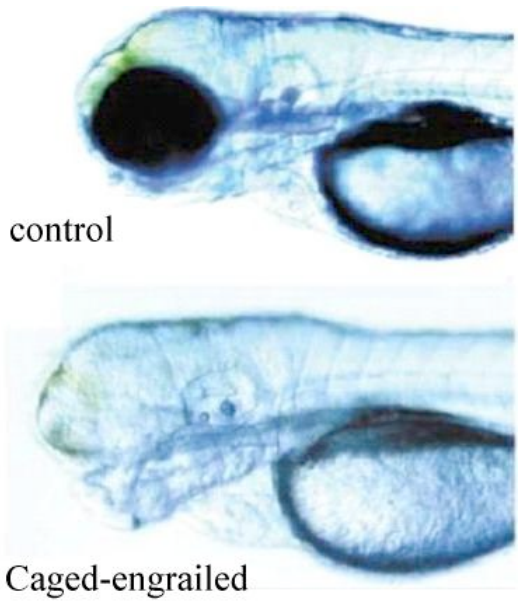

Fig. 3 Zebrafish embryo injected at the one cell stage with a caged mRNA for the transcription factor engrailed and photo-activated 12 hours post-fertilisation exhibits an absence of eye development, with permission from Ando *et al.*[38].

The problem with that approach is that the caged oligo-nucleotides have to be injected at the one cell stage. As they are diluted and degraded during the normal development of the organism, their uncaging is efficient only for a few hours after fertilization.

5 Optical control of an endogenous morphogen: retinoic acid

The spatio-temporal control of the concentration of biomolecules is particularly attractive for the investigation of developmental networks. In these cases, cellular differentiation is determined by the interaction between gradients of morphogens and signaling molecules that directly or indirectly tune the expression of various genes. It has recently been shown that these morphogen gradients determine the fate of cells with single cell spatial resolution [41]. Therefore, controlling morphogen concentration at the single cell level may allow us to study signaling and developmental networks with unprecedented spatio-temporal control and resolution. However, the quick spatio-temporal control of these endogenous factors imposes strong constraints on their possible caging. Thus it might be impossible to cage a secreted protein morphogen that has to undergo post-translational modifications or get exported via a specific pathway. In this respect retinoic acid (RA) is a target of choice for this type of investigations [42]. RA is a small lipophilic molecule whose metabolism is tightly regulated. It is synthesized from retinol (vitamin A) and can be sequestered or degraded in the cell by various proteins. As a morphogen, it is playing a role in many early developmental pathways such as somitogenesis, hindbrain development, left-right symmetry, eye and heart development [43]. Retinoic acid is easy to cage by reacting it with the alcohol of the caged moiety. The resulting ester is soluble, non-toxic, permeates the embryo and can be uncaged to exhibit the same teratogenic effects as RA, see Fig.4 [44].

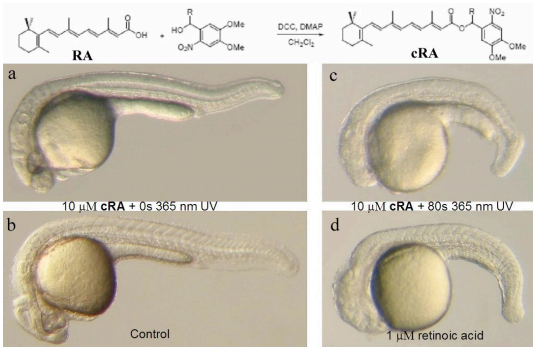

Fig. 4 Caged retinoic (cRA -formula on the right) and its effects on the development of a zebrafish embryo. In absence of UV illumination (a), it has no effect - the embryo develops normally as in the control (b). When illuminated 80 s with a 356 nm UV light, RA is released and the embryo exhibits similar developmental defects(c) as an embryo incubated in a similar concentration of RA (d).

To demonstrate the usefulness of cRA in the study of developmental pathways, we have used two-photon excitation to release RA in a few (4) cells of the dorsal part of the retina of a zebrafish embryo at the 4-14 somite stage. We chose that pathway because previous experiments with RA-soaked beads implanted in the dorsal part of the retina showed that the eye developed abnormally. In these experiments however, the whole retina is continuously subject to a high concentration of RA, whereas in our approach a pulse of RA is released in a few cells of the retina. Surprisingly however, this pulse of RA is sufficient to induce a malformation of the

illuminated retina, see Fig.5. How this local perturbation of the RA morphogen gradient is transmitted to the other parts of the retina is an interesting issue that can now be addressed in greater detail (using RA-responsive GFP embryos and quantitative RT-PCR). Beyond the study of RA in eye development, caged RA could also be used to investigate the role of RA in somitogenesis and hindbrain development. During somitogenesis, it has been proposed that successive somites are being generated by coupling the signal of a putative "somitogenesis clock" to a bi-stable switch formed by the interaction between gradients of RA and a growth factor (Fgf8) [45]. If this mechanism is correct, one should be able to generate extra-somites by activating RA at appropriate posterior regions of an embryo undergoing somitogenesis.

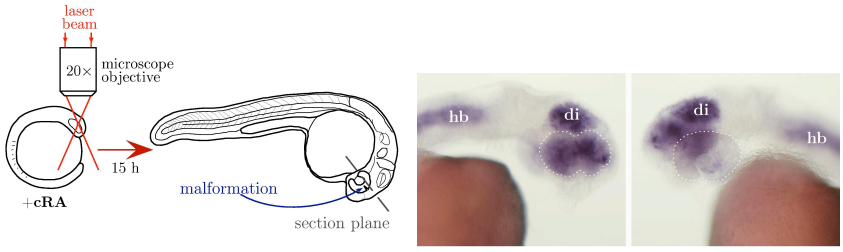

Fig. 5 (a) A pulsed Ti-Sa laser (750 nm) is used to uncage caged RA in a single cell of a live zebrafish embryo. The effect of this perturbation to the local concentration of RA is observed 15 h later. (b) Comparison of the right (illuminated) retina with the left (untreated) one. The dorsal part of the treated retina is deformed and the distribution of a retina marker (pax6, blue color) differs markedly from that of the normal (left) eye (di: diencephalon, hb: hindbrain).

6 Optical control of protein activity

The direct caging of peptides and proteins has been achieved by many different groups [7], but rarely at the single cell level in a live organism [46]. These caged proteins have residual activity when caged, incomplete recovery of activity after uncaging and most importantly they must be micro-injected into the cells.

Instead of adopting this approach, we decided to control the activity of a protein through its fusion to a steroid hormone receptor ligand binding domain. In absence of its ligand, the receptor forms a cytoplasmic complex with the heat shock protein hsp90 which inactivates the fused protein [47]. Steroid hormones are small lipophilic molecules that can diffuse through the epithelial layer that surrounds the embryo. As a result, activation of the protein can be achieved by incubation of the embryos in a standard culture medium containing the hormone of choice. This system has been used to induce the activity of a large number of proteins (transcription factors like engrailed, otx2, Gal4, p53, kinases such as raf-1 and Cre and Flp recombinases). In particular, it has gained wide acceptance as a means to irreversibly

induce the expression of a gene using a Cre-recombinase fused to a mutated steroid receptor specific for tamoxifen (ERT) [48]. In the presence of the ligand, an upstream segment flanked by loxP sites is excised allowing expression of the gene of interest. To target that approach to a single cell, we have developed a caged analog of tamoxifen (caged cyclofen) which does not isomerise upon illumination like tamoxifen and which is as active when uncaged. By using two-photon illumination, we can uncage this compound in a sub-cellular volume of a live zebrafish embryo. As shown in Fig.6, the fusion of a Cre-recombinase with this receptor performs as expected. It can be released by two-photon illumination in a few cells of a zebrafish embryo, where it removes a GFP gene flanked by two loxP sites thereby turning on a RFP gene in the target cell(s) and its descendents. That method could be very useful for cell lineage by labeling cells in an embryo and following their descendents. It might be particularly interesting to investigate tumor growth and identify the stem cells in a regenerating tissue.

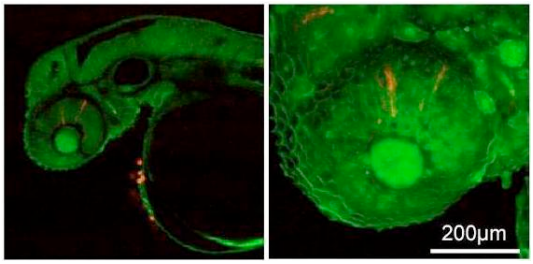

Fig. 6 Single cell activation of a Cre-recombinase fused with the ERT receptor upon two-photon release of its caged ligand. The transgenic embryo expresses a GFP gene flanked by two loxP sites. When these are excised, a dsRed gene is expressed in the target cell and its descendents, the red cells in the eye of this embryo shown at two different magnifications.

In conclusion, we hope to have convinced our reader that the optical control of the activity of biomolecules (neurotransmitters, ion channels, enzymes, transcription factors, morphogens, mRNA, etc.) opens up a new and exciting field of research, here called single cell physiology, where the investigation of physiological networks (implicated in processes such as memory, development, cancer growth, etc.) can be performed at the most relevant level for an organism: the single cell.

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