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# Regulation of Cellular Molecular Signaling by Light

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(*Invited Paper*)

**Abstract**—Laser technology has been promoting various microscopy methods and thus making great progresses in life science. Further than contribution to "seeing is believing", lasers have also demonstrated their capacity of manipulating cells and even molecular signaling. Specifically, with advances of lasers and combination with other techniques, recent reports show that cell calcium ion, a universal intra- and inter-cellular messenger, can be modulated by lasers at different levels of biological organization from organelle to tissue. It is very encouraging that laser irradiation can activate or control plenty of corresponding cell processes and functions by regulating cell calcium signaling pathways, with promising potential in both scientific research and clinical application. In this paper, optical techniques for regulation of cell calcium signaling are specifically reviewed. Most methods need exogenous chemicals or genetic materials to convert incident photon into stimulation that cells can response with specific molecular dynamics. The only all-optical approach is achieved by nonlinear excitation with femtosecond laser, despite lack of specificity and controllability, providing possibility of a totally noninvasive method without any biochemical materials and thus further potential clinical application in human beings. The developments and techniques of those methods are introduced and explained, with analysis on their properties and current challenges. Potential applications and prospective development are also discussed. Researchers on biophotonics and related biological fields can benefit from this review. It also provides a systematic reference to doctors and researchers who are working on practical application of those methods.

## 1. INTRODUCTION

The initial technical point of modern life science starts from optical microscopy. Delivered by light, biological information can be acquired with a resolution of diffraction limit. Fluorescent microscopy, one of the most basic equipment in any biological laboratory, provides researchers with insight of molecular dynamics in cells. With advances of laser technology, various microscopy techniques have been developed and making great progresses in biology research. A remarkable milestone is super-resolution microscopy, awarded by Nobel Prize in 2014, which can even break the diffraction limit by optical modulation of fluorescence. Generally, light microscopes play the role of "eye" for people to observe the micro- and even nano-world of cells. But lasers can function beyond an eye. Ashikin and Dziedzic firstly showed that lasers could work in a manner of optical tweezer to manipulate suspending cells as a "hand" [1]. Further femtosecond laser techniques could function as a sub-micron optical lancet to achieve precise surgery of direct photodisruption on subcellular structures [2, 3]. More than those pure physical manipulations, in the last two decades, series of studies presented that lasers could even

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modulate cellular molecules [4–6]. The prospect is exciting that all cellular elements, including cell structures, organelles, and signaling molecules, can be manipulated by lasers at real time while being imaged with also lasers — if the "eye" and "hand" are coordinated. This kind of microscope system is of great potential to advanced development of cell and molecular biology with powerful functions of both "eye" and "hand" simultaneously.

Direct physical manipulation of molecules by laser in live cells like optical tweezer has been impossible to date. But laser excitation may influence the state and dynamics of them directly or indirectly. Cellular molecules, including macromolecules such as proteins, nucleic acids and carbohydrates, as well as micro-molecules such as some ions and free radicals, form large number of signaling pathways to support intra- and inter-cellular communication system, which specifically or associated with other pathways regulate different cell functions and processes [7]. Those signaling pathways together finally form a complicated but ordered signaling network, enabling cells to live, develop, proliferate, and die [8]. Interestingly, most of those cell behaviors are involved with a universal signaling molecule, calcium. In living cells, calcium acts as a ubiquitous second messenger and consequently involves in most cell signaling [7]. Therefore, technology of calcium modulation is the key to investigate most cell processes and even directly control them [9]. Different from traditional approaches for cellular calcium modulation including mechanical stimulation [10], electrical stimulation [11] and chemical stimulation [12, 13], photostimulation holds the natural properties of lasers, which are controllable, precise, and most importantly, noninvasive, for biological studies. In this review, different optical cell-calcium regulation methods, including the principles and techniques, are introduced, analyzed, and discussed. Specifically, the technique of photostimulation by femtosecond laser, which is possible to provide all-optical modulation of cellular molecules without any exogenous materials or gene engineering, is presented in detail. Possible mechanism, potential application and further development of each optical technique are also prospected. We believe that this review will clarity the development and characters of those optical methods for cell molecular signaling modulation. Researchers in biophotonics and related biological or medical fields can benefit from this review to get better understanding of such optical technologies and inspiration of their practical application.

## 2. BRIEF INTRODUCTION TO $Ca^{2+}$ SIGNALING IN CELLS

Calcium, a universal messenger, plays an important role in cell signaling, which is physiologically and pathologically involved in most intracellular processes including exocytosis, contraction, metabolism, transcription, fertilization and proliferation [7, 14], and numbers of intercellular interactions [15]. Specifically, cells recruit calcium pumps, channels, sensors, and large amount of other molecules in calcium signaling pathways to regulate cellular calcium level spontaneously for different intracellular and intercellular behaviors.

Generally speaking, the components of $Ca^{2+}$ signaling can be divided into three parts, as shown in Fig. 1. Firstly, various pumps and exchangers keep reducing $Ca^{2+}$ concentration in cytoplasm. Secondly, various $Ca^{2+}$ channels can form local or global $Ca^{2+}$ elevation of various spatial and temporal dynamics by $Ca^{2+}$ release from intercellular stores or $Ca^{2+}$ influx from the extracellular medium. Those $Ca^{2+}$ elevations can be generated for two reasons: spontaneous activation of intracellular specific processes or responses to extracellular stimulations (including intercellular molecular signaling and other physical or chemical stimulations). Inositol-1,4,5-trisphophate receptors ($IP_3Rs$) and ryanodine receptors (RYRs) are the most major calcium regulators of internal stores to activate release of intracellular $Ca^{2+}$ stores. It should be noted that intracellular $Ca^{2+}$ store release is closely related with extracellular $Ca^{2+}$ influx. Store-operated $Ca^{2+}$ channels (SOC) are actually very important proteins for regulation of $Ca^{2+}$ influx based on intracellular $Ca^{2+}$ store, by which some $Ca^{2+}$ channels in membrane can be open if $Ca^{2+}$ store is released. Thirdly, the high cellular $Ca^{2+}$ level itself can stimulated a variety of $Ca^{2+}$ sensors [16–18] for more $Ca^{2+}$ release and activate specific downstream cellular processes. For example, a lot of gene transcription and expression can be activated by $Ca^{2+}$ release. Its oscillations can even increase the efficiency and specificity of gene expression [13]. Notably, organelles can also contribute to $Ca^{2+}$ regulation and be influenced by it. Mitochondria, a very important $Ca^{2+}$ regulator, are involved in transporting cytoplasmic $Ca^{2+}$ back to endoplasmic reticulum (ER), accompanied by enhanced ATP generation, reactive oxygen species (ROS) release, and even cell death [19–23]. The general effect of

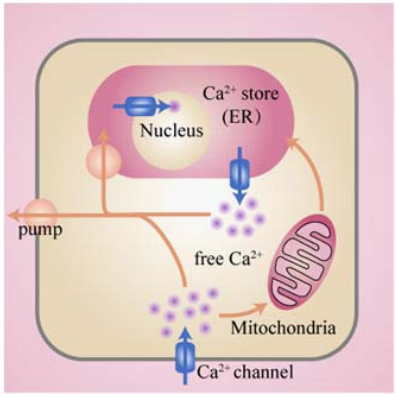


**Figure 1.** General mechanism of cell $Ca^{2+}$ store and regulation. Normally most $Ca^{2+}$ is stored in intracellular $Ca^{2+}$ stores like ER. $Ca^{2+}$ pumps can pump free $Ca^{2+}$ in cytosol into $Ca^{2+}$ stores or out cytoplasm membrane. Types of $Ca^{2+}$ channels can be activated for $Ca^{2+}$ release/influx under certain stimulation or signaling. Organelles like mitochondria are also involved in cellular $Ca^{2+}$ regulation.

those three $Ca^{2+}$ modulation parts provides a background of relatively low $Ca^{2+}$ level in cytosol and nucleoplasm at rest while rapid $Ca^{2+}$ level elevation after activated. Usually the $Ca^{2+}$ concentration in cell buffer ($\sim$ mM) is much higher than it in cytosol ($\sim$ 10–100 nM).

## 3. OPTICAL REGULATION OF CELL SIGNALING MOLECULES

Photons can initiate change of $Ca^{2+}$ concentration in naturally light-sensitive cells like cone and rod cells of vertebrate [24, 25], special plant cells [26], phototactic flagellates [27], and some archaea and bacteria [28]. After excitation, photoreceptor proteins in those cells can directly or indirectly influence calcium channels to regulate cellular $Ca^{2+}$ level. Then a subsequent question comes: can light work in other ordinary cell lines?

### 3.1. Optical Activation of Cell Calcium Mediated by Exogenous Chemicals

A natural and direct idea is to add photosensitizer (PS) into cells to make cells light-sensitive. In 1990s, with progresses of photodynamic therapy (PDT), different types of PSs were developed and used in cells for killing them by light. It was soon reported for many times that under green/red light (500–635 nm) or blue light (360–400 nm) irradiation a single phasic increase of cytoplasmic $Ca^{2+}$ lasting for up to tens of minutes was generated in different cell lines with different PSs [29–41]. The mechanism was believed as photoactivated PSs could induce $Ca^{2+}$ influx, $Ca^{2+}$ release and/or activation of ion exchange through the oxidative stress from PSs [42], as shown in Fig. 2. By applying this technique to cells which could generate intrinsic cytoplasmic $Ca^{2+}$ spiking after physiological stimulation, such as pancreatic acinar cells and rat adrenal chromaffin cells, recurrent spikes were also achieved [43]. But the side effect of PSs, which is high oxidative stress to kill cells, the very original purpose of PSs, greatly limits the application of this method.

A similar idea is to introduce calcium rendered biologically inert by photon-sensitive chemicals into cells. When illuminated by UV light, the structure of those chemicals will be modified to liberate free $Ca^{2+}$. This so-called "uncage" process can release $Ca^{2+}$ in a direct, simple, and somehow exclusive manner to generate a short spike with durations of several tens to hundreds milliseconds, in which probably few extra molecules or processes are involved, as shown in Fig. 3. This method has been used to investigate the elementary nature of $Ca^{2+}$-induced $Ca^{2+}$ release mechanism [44], the process of $Ca^{2+}$-dependent exocytosis [45], the phenomenon of $Ca^{2+}$-sensitive glutamate release [46], the ability of local calcium transients to regulate the spontaneous motility of dendritic filopodia [47] and other $Ca^{2+}$ relative processes. Similar but indirect methods utilizing other caged molecules like caged neurotransmitters for $Ca^{2+}$ release were also developed [48, 49]. Combined with two-photon excitation photolysis, spatially-

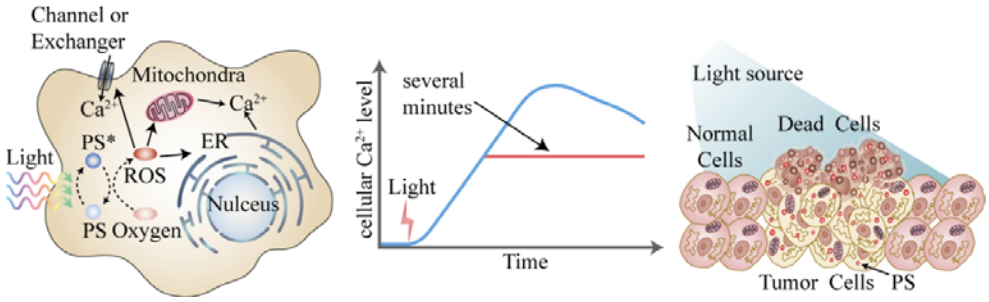


**Figure 2.** $Ca^{2+}$ release by light mediated with PS. (a) The oxidative stress from PS excited by light can stimulate $Ca^{2+}$ stores, Ca2+ channels, or organelles to release $Ca^{2+}$. (b) Representative $Ca^{2+}$ release after a certain duration of light illumination by this method. (c) Light illumination can lead to death of tumor cells treated with PS.

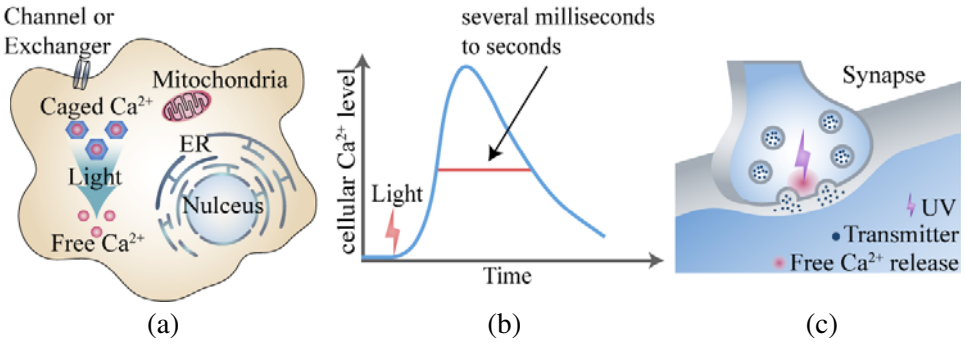


**Figure 3.** Uncaging $Ca^{2+}$ by light. (a) Caged $Ca^{2+}$ can be directly released by photochemical excitation to the inert molecules that cage $Ca^{2+}$. (b) Typical $Ca^{2+}$ change pattern of optical uncaging $Ca^{2+}$. (c) This method has been used to control neurotransmitter release by light in synapse.

confined artificial $Ca^{2+}$ sparks could be produced and used to investigate calcium-relative processes such as fundamental calcium release dynamics [50] and calcium signaling in the nucleus during apoptosis [51]. What's more, this method for high spatial-resolution calcium release could even be applied in vivo [52]. However, it should be noted that the relatively low two-photon absorption cross-section greatly limits the effectiveness and efficiency of two-photon uncaging. The difficulty in delivery of caged $Ca^{2+}$ compounds in vivo also hinders effective application to living animals [49, 53].

### 3.2. Cell Calcium Modulation by Optogenetics and Thermogenetics

The stability and biological safety of those exogenous photosensitizers and caged chemicals in cells are largely out of control. It will be much better if the gene of photosensitive proteins can be transfected into cells whose corresponding proteins can be expressed and then respond to photons to modulate $Ca^{2+}$ level. This idea was thus named as optogenetics, and now it has been developed as the most powerful tool to stimulate cells, especially in neuroscience to control neural excitability. In 2003, Georg Nagel et al. firstly reported channelrhodopsin-2 (ChR2), a single-component light-activated action channel from unicellular algae which could generate photoreceptor currents carried mainly by $Ca^{2+}$ under physiological conditions, was possible to simply depolarize cells by light illumination [54]. Soon in 2005, Deisseroth's and Nagel's groups demonstrated the optical modulation of membrane potential in excitable cells of the nematode Caenorhabditis elegans and neurons transfected with mutated ChR2(gf):YFP fusion-protein [55, 56]. Those cells showed perfect corresponding electrophysiological signals of millisecond resolution to blue light (450–490 nm) stimulation which initiated the membrane currents through ChR2, as shown in Fig. 4. Afterwards, optogenetics was combined with synthetic $Ca^{2+}$

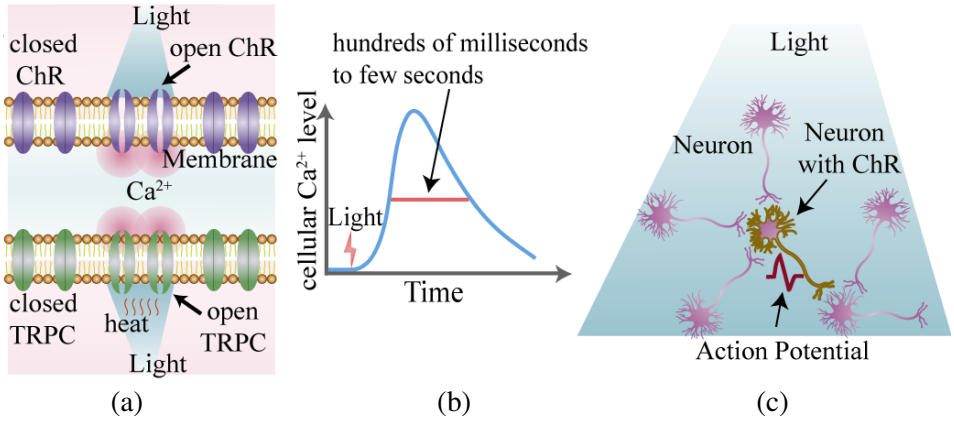


**Figure 4.** Scheme of $Ca^{2+}$ modulation by optogenetics or thermogenetics. (a) Genetically encoded light-sensitive (top, ChR) or temperature-sensitive (bottom, TRPC) cation channels can be expressed in cell membrane, which can response to direct light illumination (optogenetics) or indirect heat generated by light (thermogenetics). The specificity, efficiency, and modulation speed of optogenetics are much better. (b) The modulation speed of $Ca^{2+}$ current through cell membrane can be very fast. (c) The most important application of optogenetics is neural signaling modulation by light.

dyes such as Fluo-5F [57], or genetically encoded calcium sensors like G-CaMP [58–60] for real-time $Ca^{2+}$ dynamics detection with a temporal resolution of around 10 ms. Generally, this technique has been developing very fast and contributing greatly to neuroscience [61–63], especially to in vivo research [64–66]. Recently, it was found that proteins from optogenetics could be activated by two-photon excitation and thus combined with two-photon microscopy system [67, 68]. Further with fluorescent readouts methods by genetically encoded calcium sensors, simultaneous cellular-resolution optical stimulation and imaging can achieved [59, 60]. However, it should be noted that cations such as $Na^+$, $K^+$ and $H^+$ were also activated due to the non-specificity of those optogenetic proteins [69].

An alternative method similar to optogenetics aims to control a special type of nonselective cation channels, temperature sensitive transient receptor potential (TRP) channels, but by thermal effect of laser irradiation. This method was thus called as thermogenetics, and took advantage of heat by illumination of near-infrared (NIR) diode lasers for several tens to hundreds milliseconds [70–74]. A. Y. Rhee et al. demonstrated the $Ca^{2+}$ spike lasting for 20 seconds could be excited in cultured primary sensory neurons after 3-sec train of infrared (IR) pulses (2-ms pulse width) [70]. However, this method is relatively non-specific. On one hand, the NIR laser heating, by water absorption to photons, actually stimulated the whole cells. For example, Y. Kamei et al. showed quantitative analysis on thermal distribution by a NIR laser at 1480 nm in C. elegans and photothermal effect induced gene expression [75]. On the other hand, TRP channels were nonselective cation channels through which other cations could also enter cells. For biological safety, the deposition of heat in the tissue by this method has to be carefully considered. Moreover, since temperature sensitive TRP channels distribute only in neurons of the peripheral nervous system, this method need transfection of temperature sensitive TRP channels gene when applied in other cell lines [72].

### 3.3. All-Optical Stimulation

It should be noted that all those techniques introduced above require exogenous material for optical regulation of cell signaling. Is it possible to activate cell signaling all-optically? Photodamage during microscopy indeed indicates that cells can be stimulated solely by photons. Along with this idea, it was found that low energy visible light irradiation with about 0.1 mw/cm$^2$ power density for seconds to minutes could induce changes of intracellular $Ca^{2+}$ concentrations in macrophages [76], sperms [77–79], lymphocytes [80], skeletal muscle cells [81, 82], human fibroblasts [83] and mast cells [84]. The cellular $Ca^{2+}$ level slowly increased during continuous laser irradiation and could maintain for minutes. A possible mechanism was that cellular primary endogenous chromophores such as mitochondrial

enzymes, cytochromes, flavins, and porphyrins were stimulated by photons to generate ROS which then provided oxidative stress to the whole cell, similar to PDT. As one of ROS damage effects, stimulation to calcium stores and even ion channels occurred and thus resulted in an increase in cytosolic $Ca^{2+}$ concentration [85]. This hypothesis was supported by experimental evidences [78, 81, 86, 87]. Photothermal effect, as another type of photodamage, can also generate slow $Ca^{2+}$ increase. For example, the heating effect of infrared laser at 1863 nm could stimulate mitochondria to induce $Ca^{2+}$ increase [88, 89].

Regarding photodamage, ultra-short pulsed lasers provided much more complicated physical and biological processes but controllable photodamage by the high peak power of each pulse. Instead of linear absorption, nonlinear optical effects dominated the stimulation mechanism to cells. More importantly, cell viability can be significantly improved compared with long-pulsed or continuous-wave lasers when the pulse duration is decreased to femtosecond level. The physical process and exact mechanism remain elusive. This issue is discussed as below.

## 4. PHOTOSTIMULATION BY FEMTOSECOND LASER

In 2001, it was reported by Smith et al. that transient $Ca^{2+}$ release could be excited by tightly-focused Ti:sapphire laser stimulation (140 fs, 780 nm, 30 mw) for a very short flash (125 ms) in HeLa cells [6]. Such photostimulation by femtosecond laser could effectively activate $Ca^{2+}$ release at any position in the targeted cell. Soon following works by Iwanage et al. proposed a hypothesis that the peak power of laser pulses was the dominating parameter for $Ca^{2+}$ increase [90], and the biological responses to photostimulation strongly depended on the stimulation position in cell with different mechanisms [91]. A study from Baumgart et al. showed possible $Ca^{2+}$ sources that could response to photostimulation, $Ca^{2+}$ influx or internal calcium stores, which were both dependent on laser parameters and the location of stimulation [92]. Generally, the $Ca^{2+}$ release can be excited immediately after photostimulation to raise a $Ca^{2+}$ spike with a duration from several to hundreds seconds. It is difficult to passively activate the decrease of $Ca^{2+}$ level, which is mostly dependent on cellular spontaneous $Ca^{2+}$ modulation. Iwanage et al. claimed that intracellular $Ca^{2+}$ increase was strongly dependent on repetition rate and speculated 10 kHz might be the lowest repletion rate to produce reliably intracellular $Ca^{2+}$ increase [93]. However, Zhou et al. observed high $Ca^{2+}$ increase during disruption of cellular structure using 1 kHz femtosecond laser [94], suggesting that such $Ca^{2+}$ release could be generated by controllable and confined direct physical damage to cells. Such damage should be confined to a tiny volume to protect the cell alive, and $Ca^{2+}$ could be released by the cell through innate biological responses to such physical damage.

### 4.1. Physical Effects of Femtosecond Laser to Cells

For continuous-wave (CW) or quasi-CW lasers, single-photon absorption of cells is the major process in photostimulation. At ultraviolet or visible range, direct damage/change to molecular bands and oxidative stress by the high-energy photons mainly determines cellular responses. Thermal effect becomes more and more significant if the wavelength moves to NIR or IR band with less and less photochemical effect. But for pulsed lasers, especially ultrashort pulsed lasers, the physical processes of photostimulation become much more complicated since nonlinear effect is involved. To be simple, we only discuss pulsed lasers at NIR band with low photon energy by which little linear photochemical effect can be induced. Multiphoton excitation plays a major role in stimulation to cells. Low-order multiphoton excitation may also provide molecular damage. High-order excitation can then induce breakdown effect. In this case, the key factor that determines nonlinearity is photon density. At the same time thermal effect also plays an important role in cell damage due to high single-photon absorption by water and heat generation in nonlinear processes.

Nonlinear processes are excited at high photon density. In cells, two-photon fluorescence (TPF), second and even third harmonic generation (SHG, THG) have been quite widely used in multiphoton microscopy with a photon density of around $10^{10} \sim 10^{11}\,\mathrm{W/cm^2}$. The high photon density is required due to the ultra-low probability of simultaneous absorption of several photons that is not in the original single-photon absorption band of the molecule. The chance can be even less if the molecular cross section is smaller. It should be noted that long-time multiphoton microscopy can also induce molecular

damage to provide stress to cells. The thermal effect of the NIR photons is also a strong stimulation.

If the photon density is even higher, more than $10^{12}$ W/cm$^2$, molecules will be ionized by several different mechanisms. Electrons in the valence band can be excited to be free by directly absorbing several photons simultaneously and/or absorbing kinetic energy from other high-energy electrons by impact process. The potential well of the electron can be also changed to be a barrier and electrons can directly pass through it to be free by tunneling. The behavior of electrons is determined by the laser power density, wavelength, the molecular bandgap, and the refractive index of the medium. If the density of free electrons achieves a high level ($\sim 10^{18}$/cm$^3$) by multiphoton ionization to form plasma, it can directly induce breakdown of any material inside, with cavity effect, shockwave, and thermal effect.

Four dimensions (one temporal and three spatial dimensions) need to be considered when discussing photon density. Temporally, the pulse width determines peak power and together with repetition rate largely influences the accumulative effect of heat. Hence it is obvious that ultrashort pulses can achieve high photon density easily with a relatively low mean power. For long-duration pulses, from nanosecond to picosecond level, the thermal effect can accumulate inside a single pulse. What's more, thermal effect can further significantly accumulate if the pulse repetition rate is high (high duty cycle). If the photon density is high enough to provide optical breakdown to the medium (to be simple, the medium of cells is set as water), the plasma generation effect will accumulate and diffuse to a large area along with the long pulse duration, especially for nanosecond pulses. Therefore, regarding biological safety issue, ultrashort pulses have two advantages: 1) high peak power at low mean power; and 2) little accumulative effect (including accumulation of thermal effect and plasma generation effect) inside a laser pulse or between pulses.

Focus of the laser beam determines the spatial photon density. Numerical aperture (N.A.) of the objective and the match between the laser beam width and the objective back aperture size are very important to the focus volume. Gaussian beam can be tightly focused if N.A. $> 0.9$, which can provide a rapid decrease gradient of photon density along with the distance from the focus center and thus greatly protect cells out of the laser focus. Generally, according to theoretical and experimental studies [95, 96], the nonlinear and thermal effect can be well confined inside the short pulse and the focus volume to femtosecond pulses, especially to pulses shorter than 150 fs, with a repetition rate at less than MHz level. In this regard, the photostimulation by tightly-focused femtosecond laser holds a precise spatial and temporal resolution, and is relatively safe to cells.

### 4.2. Biological Mechanism Study

After the photostimulation by femtosecond laser to cells, series of cell processes take place as responses to the photostimulation. At the very beginning, Smith et al. proposed three possible mechanisms for intracellular $Ca^{2+}$ increase by photostimulation in their pioneering work [6]: 1) direct photodisruption or photodamage to cellular internal $Ca^{2+}$ stores followed by $Ca^{2+}$ release; 2) transient creation of a temporary hole in cytoplasm membrane followed by $Ca^{2+}$ influx; 3) indirect stimulation including mechanical force by laser-generated shockwave, as summarized in Fig. 5(a). Iwanaga et al. used two different extracellular solutions either containing $Ca^{2+}$ chelator EGTA or inhibitor for cellular sensor to shockwave-based mechanical effects to demonstrate that femtosecond laser-induced cellular calcium increase was due to the leaking of $Ca^{2+}$ through the destruction of intracellular $Ca^{2+}$ stores [97]. Zhao et al. directly photodisrupted plasma membrane of astrocytes to make a temporary hole for extracellular calcium influx [98]. And Zhou et al. reported that calcium increase in living olfactory ensheathing cells by 1 kHz femtosecond laser was related with shockwave-induced mechanical force [94]. However, the main cellular $Ca^{2+}$ store, ER, is actually conjunct with cytoplasm membrane. Therefore, it is impossible to totally prevent ER from being stimulated when cell membrane is disrupted. In fact, considering the general distribution of cellular $Ca^{2+}$ stores [99], there is little chance to keep the laser focus away from them. There also remains possibility to evoke cell $Ca^{2+}$ response by molecular deploymerization through multiphoton excitation [94]. It should be noted that there is still no report of wavelength-dependent $Ca^{2+}$ release induced by femtosecond laser, which may further implies specific innate cellular molecules able to response directly to photons for $Ca^{2+}$ modulation.

With an immediate $Ca^{2+}$ release (maybe a very localized $Ca^{2+}$ release at the laser focus) along with photostimulation, some subsequent cellular processes of $Ca^{2+}$ release are also activated. One of the most common processes is calcium-induced calcium release (CICR), a self-amplifying function for a local

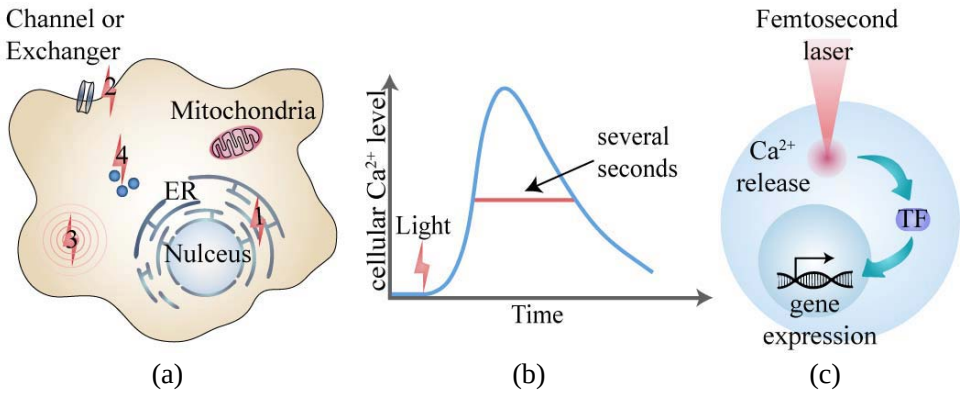


**Figure 5.** (a) Possible mechanism for intracellular $Ca^{2+}$ increase by photostimulation: 1) direct photodisruption or photodamage to $Ca^{2+}$ stores; 2) transient creation of a temporary hole in cytoplasm membrane; 3) mechanical force by laser-generated shockwave; 4) molecular deploymerization through multiphoton excitation. (b) Representative $Ca^{2+}$ release after a flash of light irradiation by this method. (c) Control of gene expression by femtosecond laser by photostimulated $Ca^{2+}$ signaling. (TF: transcription factor).

$Ca^{2+}$ spike to stimulate other $Ca^{2+}$ stores to release more $Ca^{2+}$. In 2012, He et al. found that calcium-release-activated calcium (CRAC) channels, a classic SOC regulator, was also activated after ER $Ca^{2+}$ depletion by femtosecond laser indicated by migration of stromal interaction molecule 1 (STIM1) to the ER-cell membrane junction, which allowed entry of extracellular $Ca^{2+}$ influx [100]. This was one of the main reasons why high cellular $Ca^{2+}$ level was induced by femtosecond laser irradiation apart from intracellular $Ca^{2+}$ store release, as shown in Figs. 5(b) and (c). Then those further released $Ca^{2+}$ diffuses away to the whole cell [101]. In this process the role of nuclear membrane is still not totally clarified. It was reported that the nuclear tubule might contribute to the diffusion of cytoplasmic $Ca^{2+}$ into nucleus [102]. Actually, $Ca^{2+}$ generation is not an exclusive cellular response to photostimulation. A lot of cell processes are activated simultaneously and some of them can feedback to contribute to more $Ca^{2+}$ release, like ROS generated by two-photon excitation of endogenous absorbers [103] and/or oxidative stress to ER [104].

Interestingly, intercellular $Ca^{2+}$ wave propagation among excitable cells or non-excitable cells was also observed after the intracellular $Ca^{2+}$ release by femtosecond laser [6, 98, 105]. He et al. proposed three possible mechanisms: 1) $Ca^{2+}$ signaling molecules such as ATP was released from the photostimulated cell and diffused away to excite $Ca^{2+}$ release in surrounding cells; 2) mechanical stress of shear flow by the plasma generation impacted surrounding cells to activate the release of $Ca^{2+}$; 3) mechanical stress of acoustic wave stimulated $Ca^{2+}$ release along its propagation [106]. Several works suggested the $Ca^{2+}$ wave from a single cell stimulated by femtosecond laser at a relatively safe power was mediated by ATP which diffused in the cell medium and activated $Ca^{2+}$ release in surrounding cells through ATP receptors on cell membrane [91, 105, 107]. A recent work by Compton et al. demonstrated the contribution of mechanical stress by a high-power nanosecond laser focused outside cell to $Ca^{2+}$ wave generation [108].

### 4.3. Subsequent Cellular Responses to Femtosecond Laser Induced Calcium Change

ROS, originally mainly leak from the electron transport chain (ETC) in mitochondria during respiration, are quite related with $Ca^{2+}$ regulation in living cells. The increase of ROS or cytoplasmic $Ca^{2+}$ can greatly influence level of the other one. Under normal condition, mitochondria will specially situate close to $Ca^{2+}$ channels to form local microsystems where released $Ca^{2+}$ from $Ca^{2+}$ channels is partially taken up by mitochondria. The abnormally high-level $Ca^{2+}$ in mitochondria promotes ROS generation, activation of permeability transition pore (PTP), and enhancement of ROS releasing to cytosol. In turn, released ROS can also activate molecules like $IP_3Rs$ and RyRs to facilitate $Ca^{2+}$ release from internal

stores [21]. Furtherly, apoptosis may be initiated by overloading mitochondria with $Ca^{2+}$ to release cytochrome $c$ through the PTP [19, 109], where ROS play a role in membrane barrier dysfunction, structural deformations and fragmentation of the nuclei and DNA strand breaks [110]. Therefore, ROS can be affected as a response to femtosecond laser and in turn induce calcium increase and thus influence relative signal pathways and cellular processes like cell death [111]. Nevertheless, there are seldom researches on detailed and in-depth subsequent cellular responses to femtosecond-laser stimulation with quite a lot of problems elusive.

### 4.4. Application of Femtosecond Laser-Controlled Calcium Change

As a high spatiotemporal-precision, non-contact and non-disruptiveness technology of cell $Ca^{2+}$ regulation, femtosecond-laser stimulation has been used to investigate and even control basic biological processes relative to cellular calcium, such as calcium store in cells [101], the role of calcium in cell apoptosis [102], cellular oxidative pressure [100], the role of mitochondria in laser-controlled calcium change [88], intercellular calcium propagation [112], photogeneration of membrane potential hyperpolarization and depolarization in non-excitable cells [113], calcium-relative gene expression control [114] and muscle cell contraction control [115]. As shown in Fig. 6, the system is very easily to set based on a microscope system.

Another important application is optical modulation of $Ca^{2+}$ in neuroscience. Hirase et al. firstly stimulated neurons by femtosecond laser to modulate membrane depolarization in 2002 [116]. In 2005, Smith et al. demonstrated femtosecond-laser stimulation could evoke $Ca^{2+}$ spikes in neural-type cells [117]. After that, this method developed fast and brought encouraging results in different neural-type cells such as GH3 cells and astrocytes [98, 107, 118, 119] and cortex slices [120]. Furthermore, Liu et al. found $Ca^{2+}$ wave could be also excited in neurons by femtosecond laser and used to identify neuronal connections, and finally gained neural circuits by this way [121]. Parys et al. investigated intercellular calcium signaling between astrocytes and oligodendrocytes via gap junctions by focused femtosecond laser to induce $Ca^{2+}$ increase in targeted cells [122]. A very promising work was reported by Zhao et al. that modulation of synchronous calcium oscillations in hippocampal neurons could be achieved by femtosecond laser stimulation to astrocytes [123].

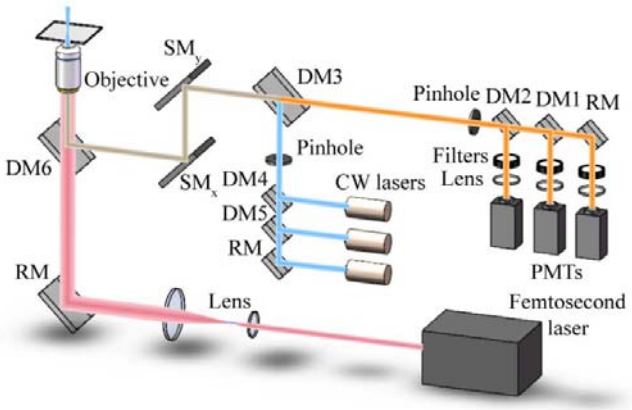


**Figure 6.** Typical system for photostimulation by femtosecond laser. The system can be set based on a confocal microscope. The femtosecond laser should be expanded and collimated by two lens and then coupled into the objective to be focused on cells. RM: reflect mirror; DM: dichroic mirror; SM: scan mirror; PMT: photomultiplier.

## 5. DISCUSSIONS

The scheme of optical regulation of cell $Ca^{2+}$ is actually very similar to fluorescence excitation. If the cells are introduced with special materials or transfected with engineered genes, the incident photons can

then excite "fluorescence" — $Ca^{2+}$ dynamics, mediated by those materials or expressed proteins. The $Ca^{2+}$ increase is actually only a by-product of laser irradiating photosensitizers which mainly generate oxidative stress to cells. The high-level oxygen can greatly damage cells directly with simultaneous or subsequent activation of various processes like $Ca^{2+}$ increase and even necrosis. Therefore the $Ca^{2+}$ release is not the original target here without high specificity.

If cells are loaded with caged $Ca^{2+}$, the specificity can be improved theoretically under the condition that the light irradiation merely uncages $Ca^{2+}$. However, since most caging chemicals only response to photons at UV range, direct single-photon excitation to cells induces many cell processes as well as photodamage. Actually illumination of UV light can directly induce $Ca^{2+}$ increase in cells by photodamage or oxidative stress without the need of caged $Ca^{2+}$. The loading of photosensitizers and caged $Ca^{2+}$ also limits application of those methods in in vivo research.

Similar to genetically encoded fluorescence, genetic material can be transfected into cells to express proteins that can response to photons to activate cation current. Nowadays construction of optogenetics plasmids is very developed and corresponding animal models are accessible. Optogenetics is now the most widespread technology of optical modulation on $Ca^{2+}$ and making great progresses in neuroscience research. The only technical limitation is the lack of spatial resolution in tissue because scattering photons also activate $Ca^{2+}$ current in genetically modified neurons out of focus. Another challenge is hard use in human due to the biological ethics and safety issue for gene engineering. Some trials have been made to take advantages of photothermal effect considering some TRP channels response to heat. But thermal effect of lasers is difficult to control and lack of temporal resolution.

Direct photostimulation by femtosecond laser without any biochemical materials or gene engineering is an alternative method to excite $Ca^{2+}$. Importantly, the photostimulation is quite clean, noninvasive, and safe to overcome the ethical and biological safety issue. But this method is unable to provide a greatly controllable $Ca^{2+}$ modulation like optogenetics so far to date, especially in the temporal dimension. The $Ca^{2+}$ spike, once excited, is difficult to be actively close. It still remains a lot of problems to be investigated especially to the biological mechanism of $Ca^{2+}$ release. The possibility of innate cellular proteins that can directly or nonlinearly response to photons for $Ca^{2+}$ modulation is quite attractive and may exist. It should be noted that significant physiological and molecular changes can be induced by femtosecond laser in previous studies on photodamage during multiphoton microscopy [110–114]. In our recent experiments, it was found that moderate and controllable $Ca^{2+}$ release could be also activated by multiphoton excitation which implied possible innate molecules inside cells that could directly response to photons for $Ca^{2+}$ modulation. It can be expected that with some further research, an all-optical method for $Ca^{2+}$ modulation with high controllability and high spatial and temporal resolution to provide $Ca^{2+}$ spikes will be developed, which will definitely promote related research and applications.

## 6. SUMMARY

Lasers can somehow modulate cell molecular dynamics by linear or nonlinear processes. Specifically, series methods of optical regulation of cell $Ca^{2+}$ have been developed, which are of great significance to the research of cell biology, neuroscience, and related biomedical fields. To regulate and enhance cell responses to photons, specified materials may need to be introduced into cells, or engineered genes should be transfected. Incident photons can then induce modulation of $Ca^{2+}$ level in cells just like single-photon excited fluorescence. By nonlinear optical processes, photostimulation by femtosecond laser can directly activate cellular $Ca^{2+}$ without any exogenous materials. However, limitations still remain in each method, especially in further in vivo research and application. Even though, those optical methods provide alternative choices for precise, fast, noninvasive, and controllable modulation of $Ca^{2+}$ that may be great challenges to traditional biochemical technologies. Beyond that, such interdisciplinary field of lasers and cells excites a lot of novel findings out of traditional systems. It can be expected that laser technology will continue to advance methodology and research in cell biology, neuroscience, and other related biological fields and hold promising potential in application of medicine.

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