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REVIEW

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Two-Photon Microscopy for Chemical Neuroscience

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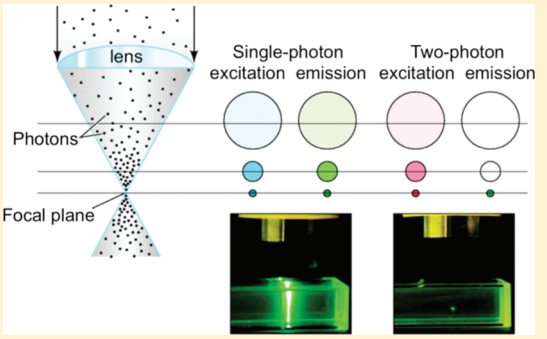
ABSTRACT: Microscopes using nonlinear excitation of chromophores with pulsed near-IR light can generate highly localized foci of molecules in the electronic singlet state that are concentrated in volumes of less than 1 fL. The three-dimensional confinement of excitation arises from the simultaneous absorption of two IR photons of approximately half the energy required for linear excitation. Two-photon microscopy is especially useful for two types of interrogation of neural processes. The first is uncaging of signaling molecules such as glutamate, because stimulation is so refined it can be used to mimic normal unitary synaptic levels. In addition, uncaging allows complete control of the timing and position of stimulation, so the two-photon light beam provides the chemical neuroscientist with an "optical conductor's baton", which can command synaptic activity at will. A second powerful feature of two-photon microscopy is that when used for fluorescence imaging it enables the visualization of cellular structure and function in living animals at depths far beyond that possible with normal confocal microscopes. In this review, I provide a survey of the many important applications of two-photon microscopy in these two fields of neuroscience and suggest some areas for future technical development.



Light has been used to study cells for over 300 years. However, until recently, only normal, linear modes of excitation have been used. The probability of such absorption is defined in the text-book equation, the Beer−Lambert law, which states that the likelihood of producing an excited state is directly proportional to amount of light absorbed throughout the irradiated specimen. Thus, when light is focused through a lens into a homogeneously fluorescent sample, the density of excited molecules in each *z*-section increases, but the total number of absorbed and emitted photons in each *z*-section is the same (Figure 1).

While normal light microscopy provides a fantastic amount of useful information about cells, their complex structures and myriad functions can be more fully appreciated when they are seen in three dimensions (3D) at high resolution.[1,2] Such microscopy (called "laser-scanning confocal microscopy") only became practical in the 1980s, since it required important new hardware (computers, lasers, and digital storage) that was being developed at that time.[3] Since the wavelength of light turns out to be on an appropriate scale for subcellular resolution of many biological phenomena, it is no exaggeration to say that the development of laser-scanning confocal microscopy revolutionized many areas of biological science.[2,3] This method still remains the workhorse of biological imaging more than 30 years later.

Two-photon molecular excitation was first used by chemists in the 1960s and 1970s to study the selection rules for electronic transitions.[4] Since the generation of the excited state is nonlinear, a two-photon laser beam focused through a microscope lens provides three-dimensionally confined excitation that is inherent to the excitation modality (Figure 1).[5,6] Its application to biological microscopy for 3D imaging in terms of proof of principle appeared first in 1990,[6] and application to live-cell imaging and uncaging started to establish the technique shortly after this (1993−1995). But it was not until lasers became "user friendly" (2003 onward) that two-photon microscopy became a standard method in the biological sciences.[7−9] And just as laser-scanning confocal microscopy revolutionized imaging in the biological sciences in the 1990s, two-photon microscopy has had substantial impact in the new millennium.[8] The focus of this review is this recent period. My intention is to provide an introduction of the method to chemists and chemical biologists who may think HeLa cells are "In Vivo". I also hope that chemical neuroscientists who work with animals using other approaches could be inspired to use this new method to study cellular structure and function in situ, since it allows extremely precise monitoring and control of (sub)cellular biochemical events.

This review is divided in two parts. The first part is an overview of the principles of two-photon fluorescence microscopy and is illustrated with examples concerning imaging cells in the neocortex of living mice. The second part reviews two-photon uncaging microscopy and focuses on the use of caged glutamate for the study of neurons in brain slices.

TWO-PHOTON FLUORESCENCE MICROSCOPY

Principles of Two-Photon Imaging. Since laser-scanning confocal microscopes provide excellent 3D images of fixed and living biological samples, what are the advantages of using

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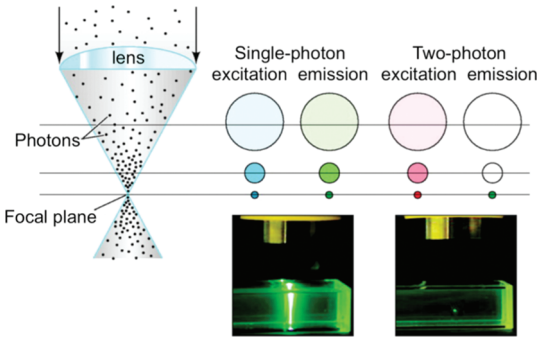

Figure 1. Comparison of single- and two-photon excitation. The objective lens of a fluorescence microscope focuses the excitation light to a spot. The cartoon of this process (left) illustrates how the photons become increasingly concentrated as they approach the focal plane. The colored circles show that along the excitation path the flux per unit area increases while maintaining the number of photons in each z section. This is true of both single-photon (blue circles, middle) and two-photon excitation (red circles, right). The linear nature of one-photon excitation thus creates the same number of excited states in each z section (green circles, middle). In contrast, the nonlinear nature of two-photon excitation produces punctate emission in the focal plane (green and white circles, right). The photographs show the emission profiles from single- and two-photon excitation of a cuvette solution of a fluorophore.

two-photon microscopy for fluorescence imaging, especially of live cells? Since two-photon imaging uses light of longer wavelengths than regular imaging (e.g., 820−900 nm vs 488 nm for green fluorophores), it is most often said to be "less phototoxic" and "provide greater depth penetration" because IR light is used.[9] While both of these advantages are real, they are not the most important ones for the success of two-photon fluorescence microscopy vis-à-vis traditional laser scanning confocal microscopy. The most significant advantage is that two-photon microscopy can collect both scattered and unscattered photons during the imaging process.[8−10] In order to understand why this is so important, the means by which these two methods make 3D images must be appreciated. Both two-photon and laser scanning confocal microscopy point the laser beam used for imaging with two independently controlled mirrors into the sample. The "x-mirror" moves the beam continuously (i.e., *scans*) in a straight line. The second "y-mirror" is used to reposition the beam for additional scans by the x-mirror. This process is called raster scanning and creates a two-dimensional (2D) image of a sample. Both methods use such raster scanning for imaging, but they differ in the means by which they create a 3D image. Since most complex biological samples are widely labeled with fluorescent markers, normal lasers will produce emission from the point of focus and volumes above and beneath this volume of interest (VOI), as seen in Figure 1. Thus, laser scanning confocal microscopy provides 3D imaging by physically blocking fluorescent signal that does not emanate from the VOI. This is done by placing a small aperture (called a "pin hole" for obvious reasons) in front of the detection system (a photomultiplier tube, or PMT). The key property to the success of this strategy, it is positioned along the optical path such that only photons from the desired VOI pass through the hole (these are called "ballistic photons"[10]). The position of the PMT and the point focus of the lens (the VOI) are referred to as being optically conjugate. Since two-photon excitation is inherently confined in 3D (Figure 1, right), a pinhole is not required and, in principle, essentially all fluorescence could be collected because all photons emanate from the targeted VOI. For many samples, the images made by laser scanning confocal microscopy and two-photon microscopy are the same, so under what circumstances does two-photon microscopy become the method of choice for biological imaging? The answer lies in the area of "deep tissue imaging".[9] All living biological samples *scatter light* to some extent, and this scattering affects both excitation and emission, but scattering only becomes significant beyond 20 μm.[11] Thus, for thin samples (e.g., cultured cells on coverslips, a fixed tissue sample from serial section analysis, etc.) confocal microscopy and two-photon microscopy produce comparable images, and sometimes the former can even outperform the latter. However, the deeper the VOI is inside a sample the more likely emitted photons are to be scattered or become nonballistic and thus to miss the confocal pinhole.[10] The absence of the pinhole in a two-photon microscope allows much more of the scattered signal to be detected, with concomitant deeper tissue imaging.[9]

Two-Photon Imaging of Transgenically Fluorescent Mice. Green fluorescent protein (GFP) was isolated from jellyfish and is spontaneously fluorescent; it integrates well into many genomes and has been used to label proteins without changing their function. It was discovered in the early 1960s, but it was not until the "molecular biology era" (1990 and later) that this had a revolutionary effect on biological sciences.[12] In 2000, several transgenic mice were developed in which populations of neurons were selectively labeled with blue, green, yellow, or red fluorescent proteins[13] (B/G/Y/RFP or generically XFP). Two of these lines have been particularly fruitful for neuroscience research, and are called H-line (a YFP mouse) and M-line (a GFP mouse). These mice are commercially available from Jackson Laboratories (H-line, stock no. 3709, and M-line, stock no. 7788). They are useful for two reasons. First, the fluorescent protein is expressed at high levels, so the labeled neurons are very bright. In the case of H-line, single spines can be imaged 500−600 μm into the brain (Figure 2). Second, only a subset of pyramidal neurons in the cortex are labeled (as well some in the retina, cerebellum, and dorsal root ganglion). Since successful imaging rests upon one simple factor, namely, *contrast*, such sparse labeling is crucial. Under such conditions, a few brightly labeled neurons can appear starkly against a relatively dark background, just like a Golgi stain, but with the additional advantage that the stain is in living cells in a living organism. Thus, in combination with the advantages of two-photon microscopy outlined above, it is possible to image the entire dendritic tree of neurons in the neocortex of living, transgenically fluorescent mice (Figure 2).

Shortly after these mice were developed, two laboratories published independently studies of spine head stability in H-line (Gan and co-workers[14]) and M-line (Svoboda and co-workers[15]) mice in the same edition of *Nature*. There is broad agreement between these two groups, especially over the stability of neuronal structures in adult mice.[16] Specifically, both find that the dendritic trees of pyramidal neurons in the neocortex do not change at all over many months and the total number of spine heads does not change under basal conditions. Even though these two groups disagree[16] somewhat[16,17] on turnover rate of spines in layer 1 of the neocortex, they still agree that a large majority of individual spines are persistent for more than 1 month (range 75−95%, depending on the report) and that many spines (50−75%) persist for much longer periods.[16] While there has been some controversy over the apparent differences between these two groups, from the overall perspective of using

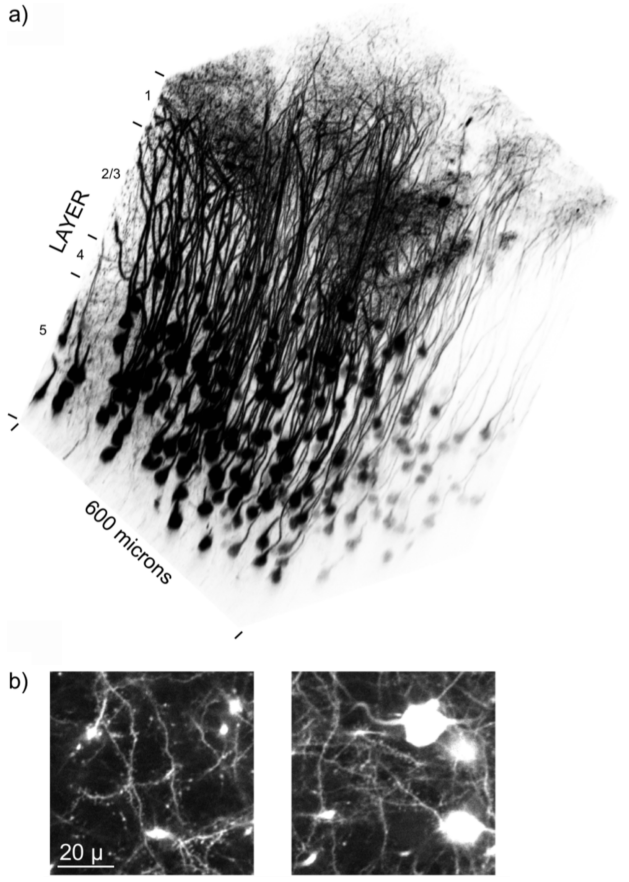

Figure 2. Two-photon imaging of H-line mice. (a) Three-dimensional reconstruction of pyramidal neurons in the neocortex of a living H-line mouse. The eYFP expression is such that the entire dendritic tree can be imaged. Even spine heads 500−600 μm below the pia can be clearly seen (b).

two-photon fluorescence microscopy to follow the fate of dendritic structures in the superficial layers of the mouse neocortex, this general agreement is important. It sets a baseline for application of this important technique to the study of two areas that are at the forefront of contemporary neuroscience, namely, how brain structures change during learning and how brain structures decay during neurodegenerative diseases. Thus, the differences might appear to be rather arcane to those outside the field, but without a basic consensus as to the general stability of brain structures under basal conditions, other laboratories could not apply this new and powerful technique to the question of how the brain changes during experience.

Two-Photon Imaging of Sensory Experience in Transgenically Fluorescent Mice. Having developed a very powerful method for noninvasive fluorescent imaging of single synapses in mouse brains, it is of no surprise that the laboratories of both Gan and Svoboda used their technique to study how experience changes brain structures. Since whiskers are so important to rodent life and the circuit from whisker to cortex is well-defined, both laboratories have also studied the effects of whisker removal on spine stability.[18,19] Thus, removal of half or all of the whiskers in one pad in the short term is reported to have little effect on spine head stability by both laboratories. There is again some disagreement about the effects of longer periods of deprivation. Gan and colleagues report only very modest effects (3% reduction in spine elimination) after two months of chessboard pattern deprivation in adult mice.[18] Svoboda and colleagues found that after 20 days the percentage of "newly persistent" spines doubled, but overall there was no significant change in spine head numbers between spared and deprived brain areas.[19]

Other laboratories have used In Vivo two-photon fluorescence microscopy of XFP mice to study much more radical interventions. Hübener and co-workers used laser ablation of a small area of the retina of M-line mice and monitored the change in spine turnover rate over 2 months.[20] They found that ablation caused a >4-fold increase of turnover of spines, without any change in total number (i.e., ca. 90% of spines were "replaced"). Hübener and co-workers have also recently made another contribution to this field. By blocking one or both eyes for certain periods in M-line mice, they could use chronic two-photon imaging to follow the consequences of such experience in a long-term fashion.[21] Using two periods of mono-ocular deprivation, they found that spines created during the first period were resistant to change in the second period, suggesting that the first experience predisposed the spines to consolidation. In a series of studies on ischemic stroke in H-line mice, Murphy and co-workers report that in severe stroke, spines are rapidly lost and dendrites swell, and remarkably, after reperfusion of occluded vessels, spines and dendrites recover their previous forms.[22−24] Such studies are a very elegant demonstration of the power of longitudinal two-photon fluorescence microscopy, since the more traditional means of having to take "time points" by euthanization cannot show recovery or replacement of lost neuronal structures, but merely the health of the population as a whole.[25]

The localization of "memory traces" are a long sought-after goal of neuroscience.[26] One idea is that there is some physical representation of memory or "engram" that actually encodes memory in the brain. Since two-photon microscopy allows longitudinal imaging of changing brain structures in the neocortex, and many think this area of the brain is the ultimate depository of many forms of memory, a few reports have recently appeared showing a correlation of changes in spines due to a specific learning task using this technique. Again, Gan and co-workers have pioneered these efforts; they used two-photon microscopy of H-line mice to show that spine heads in the primary motor cortex are specifically stabilized during learning tasks.[27] An equally compelling study came from Zuo and co-workers, who showed that during learning to grasp and eat with one forelimb, only spines in the contralateral motor cortex were stabilized.[28] No significant spine stabilization was detected in any of the surrounding cortical areas (over 33 000 spines were imaged in this study!). Finally, even though transgenically fluorescent species other than mice have been difficult to make, viral infection of song bird cortex with GFP allowed In Vivo two-photon fluorescent imaging of spine head consolidation during song tutoring.[29] This remarkable study showed that large mushroom spines were specifically stabilized during a crucial learning task for young birds (song tutoring), suggesting that large spines are indeed "the physical traces of long-term memory".[30]

Imaging of Learning and Memory Using Two-Photon Excitation of Calcium Indicators. Imaging XFP transgenic mice gives structural correlates of rodent experience in the subset of labeled neurons. Such changes are only one form of the "engram of learning". Electrical activity has been humorously dubbed the "other side of the engram",[31] and one would expect that changes in the electrical activity of neurons, either individually or collectively, also encode memory. Recently, several

laboratories have started to use In Vivo two-photon imaging of neuronal Ca^{2+} to follow electrical activity. Of course electrodes have been used for many years to follow changes in neuronal action potentials and circuit activity. This classical approach has many attractive features in that it can measure electrical activity at many levels (single cell patch clamp, field potentials, population spiking, wave activity, etc.) in any volume of the brain. Chronic studies in freely moving rodents are routine. Electrical measurements give direct access to the most important neuronal function (action potentials), which they measure with superb temporal fidelity. High-resolution imaging does offer one important advantage over all these features, which many still think makes the technique useful for some questions: it allows one to ascribe activity directly to single visualized neurons. Even if one measures single neurons with electrodes In Vivo, it is very difficult to locate the position of these cells with any real precision post hoc. Using organic calcium dyes, Svoboda and co-workers have used two-photon imaging of neuronal calcium in awake mice to start to understand how subsets of neurons in the neocortex respond to learning and sensory experience. They used a multiple trial-learning paradigm in a head-fixed, lick/no-lick task.[32] The water reward was paired with an odor, and the withholding of water with another odor. Hundreds of trials over a few days encoded a selective neuronal firing pattern in the motor cortex. Interestingly, within the field of view, one set of neurons fired during lick reward, and another during correct refusal, whereas the majority of neurons were not active during either experience. In other studies of awake mice, Tank and co-workers used genetically encoded Ca^{2+} indicators[33] (GECIs) to follow the electrical activity of hippocampal neurons during negotiation of a "virtual maze". In this technical tour de force, they could image Ca^{2+} in single neurons that consistently responded to the experience of the virtual maze.[34] The advantage of GECIs is that they have the potential to allow repetitive reimaging of experience. Indeed, their promise has been shown by two other groups in this regard in the visual cortex[35−38].

Two-Photon Imaging of Disease in Transgenically Fluorescent Mice. Even though Alzheimer's disease (AD) is the most prevalent cause of neurodegeneration among the elderly (ages >70 years) and was first described over 100 years ago, its genetic basis has not yet been uncovered.[39,40] However, a number of rare cases of what is now called "early onset" or familial AD (FAD) have been discovered in the past 20 years. Since the pathological hallmarks of FAD are the same as AD (i.e., amyloid plaques, neurofibrillary tau tangles, and neuronal atrophy), the protein mutations that cause FAD have been used to model the disease in transgenic mice.[41] These are widely thought of as models of AD, so a large number of such mouse lines have been made. Of particular interest here are a few such FAD mice that have been labeled with fluorescent proteins. With these tools, two-photon imaging of XFP/FAD mice has proven to be a powerful technique for the study of neurodegeneration (we might call these mouse models of "engrams of forgetting").

The initial studies have been, not surprisingly, of the two best-characterized FAD mouse models (called PDAPP[42] and Tg2576[43]). However since neither of these mouse lines were already known to show any profound neuronal atrophy, the reported changes in neurons seen using two-photon imaging were extremely modest, with at most only a few spines being lost or dendritic branches undergoing "a change in curvature". Even creating a bigenic mouse from crossing H-line and the APPswe/PS1d9 Alzheimer mouse (a more recent model, with more aggressive plaque formation) did not yield substantial changes in neuronal morphology, only small local changes.[44] A recent, elegant two-photon longitudinal imaging study of a *trigenic* FAD mouse did show significant neuronal loss for the first time. Herms and co-workers saw a small percentage of layer 2/3 YFP-labeled neurons disappear from 3xTgAD mice[45] in young (4−6 months) but not older mice. Since no neuron loss had been reported in this mouse previously, this result was very striking.[46] Labeling microglia with GFP revealed that the presence of the fractalkine (or CX_3CRL1) receptor was essential for neurons vanishing. A subsequent comprehensive report from the Herms group of the 3xTgAD-YFP mouse revealed that this mouse model of FAD had several parallel modes of dendritic spine loss.[47] In an equally elegant study, the same laboratory have published a study of a prion protein model created by injection of prion protein into the neocortex of H-line mice.[48] Longitudinal imaging revealed that dendrites are the first structures to undergo significant atrophy (seen as blebbing), followed by nearby spine heads being lost in such mouse models of this disease. Thus, in mouse models of neurodegeneraion, two-photon microscopy can supply, via time-lapse imaging, a uniquely powerful view of neuronal atrophy if appropriate models are used as subjects.

Two-Photon Imaging of Transgenic Mouse Models of Disease with Exogenous Labels. In principle, it is much easier experimentally to add an organic dye or stain to neuronal tissue for imaging than to use genetically encoded stains. Indeed, modern neuroanatomy is built upon this foundation. Thus, the very first (published 1 February 2001) longitudinal, two-photon imaging experiment was using a classic fluorescent plaque dye (thioflavin-S) to reimage amyloid-β plaques in Tg2576 mice.[49] Since thioflavin-S does not cross the blood−brain barrier, the dye was injected into the brain by inserting a small needle through the skull immediately prior to imaging; this method allowed Hyman and co-workers to reimage plaques at high resolution for the first time. Gan and workers have used the same approach to study dendritic stability in another Alzheimer mouse.[50] In these experiments, they crossed the PSAPP and the H-line mice and injected Congo red into the brain through a small crack made in the skull. This study showed that many (11 of 30) of the YFP-labeled neurites that could be imaged over 4−5 weeks that were close (inside a 15 μm shell beyond the plaque) to a plaque volume fragmented and disappeared during the imaging period.

Since injecting a water-soluble dye is technically challenging, Klunk developed a plaque dye that crosses the blood−brain barrier. This dye, called methoxy-XO4, is soluble in DMSO, can be injected IP into mice,[51] and has been used by several laboratories to perform longitudinal plaque imaging using two-photon microscopy. In particular, the team of Hyman and Bacskai and their co-workers have pioneered the use of methoxy-XO4 to follow plaques over time in several elegant studies.[43,44,52] Importantly, they studied plaques in bigenic mice, that is, H-line mice crossed with either Tg2576 or APPswe/PS1d9 mice,[44] and found rapid appearance of plaques (between daily imaging sessions), which was closely associated with blebbing of adjacent YFP-labeled dendrites. However, only 26 new plaques were detected in 14 animals in 1285 imaging sessions, and only 10 dystrophic neurites were associated with these plaques. This study suggests that while longitudinal two-photon imaging seems ideally suited to the study of disease-driven neuronal atrophy, its real success in producing imaging of dendritic decay is predicated upon selection of robust mouse models of neurodegeneration.[45−47]

Two other groups have recently reported apparently different results when studying plaque deposition in the Tg2576 and APPswe/PS1d9 mouse models of FAD. Jucker and co-workers used careful window implantations into APPswe/PS1d9 mice to detect slow plaque growth over 25 weeks of longitudinal imaging of individual mice.[53] Herms and co-workers report similar findings in the Tg2576 mouse.[54] Both groups report faster plaque growth in younger mice and suggest that this can only be reliably detected as a result of imaging mice for several weeks. As an important control, Jucker and co-workers studied the effects of window implantation in the Iba1-eGFP mouse on microglia activation. In contrast to previous reports,[55,56] using their approach they detected no significant gliois. In another study, the same group of researchers had examined how microglia were activated by the appearance of neurotoxic insults in the CNS, namely, amyloid plaque deposition.[57] Two-photon imaging of Iba1-eGFP/APPPS1 mice revealed that after plaques appeared, microglia were seen to migrate to them and often internalized the plaques, suggesting that stimulation of this mechanism might be a therapeutic avenue for treatment of AD. A recent study from Grutzendler and co-workers confirms the importance of microglia in amyloid disruption in the TgCRND8 model mouse of FAD in which they saw microglia internalize protofibrillar amyloid-β but not congophilic plaques.[58] It should be noted that there is an important technical problem with plaque reimaging with exogenous dyes. It is impossible to guarantee the same level of staining of every plaque for every application of dye. So defining precise plaque size In Vivo is fraught with difficulties. Second, using two-color imaging of dyes that overlap in their spectral output (e.g., Congo red or methoxy-XO4 and YFP) makes the edges of plaques difficult to define precisely. Having said this, two-photon imaging studies imply that plaques are toxic species toward neurons and therefore suggest that their dissolution remains an important therapeutic target.[59,60]

The effects of AD on calcium signaling in neurons and astrocytes have also been studied In Vivo using two-photon imaging. In these chronic experiments, organic dyes (e.g., OGB-1, fluo-4, or X-rhod-1) are loaded into astrocytes or neurons in transgenic AD mice. Three laboratories have reported hyperactive calcium signaling in layer 1 of the CNS. In a new mouse model, Konnerth and co-workers showed that neurons near (<50 μm) plaques are hyperactive.[61] In APPswe/PS1d9 mice, Bacskai and co-workers found that spontaneous (some prefer "intrinsic"[62]) intracellular astrocyte signaling was also hyperactive[63] (a 3-fold increase compared with WT or young AD mice), but unlike the report by Konnerth's lab, there was no spatial correlation with plaques. Both of these mouse models have presenilin-1 mutations that perturb calcium buffering by Ca^{2+}-ATPases[64], as well as the IP_3-R open-channel probability.[65] In contrast, an earlier mouse model has no PS1 mutations, and this mouse has also been reported[66] to have hyperactive signaling and intercellular astrocytic calcium waves, suggesting that disruption of IP_3-R is not required for hyperactive calcium signaling In Vivo in FAD mice.

Suggestions for Future Developments for In Vivo Two-Photon Imaging. High resolution In Vivo neuronal imaging that was revolutionary in 2002[14,15] has become "routine". Such imaging remains limited to the superficial layers of the brain (Figure 2). Two-photon imaging would be even more valuable if it could be applied to other brain regions that lie in deeper areas. Fluorescence microendoscopy offers some promise in this regard.[67,68] It has only been implemented by a few laboratories and requires the removal of a small part of the neocortex, yet it seems the only way to deliver and collect light from very deep brain tissue without removing substantial portions of the neocortex.[34,69] Thus, it may be a technique that will attract more development over the next few years.

While the development of eGFP and eYFP by Tsien and co-workers in 1994 was not undertaken with two-photon microscopy in mind, it turns out that these fluorophores are well suited to two-photon excitation[7]. Their subsequent integration into the mouse genome has provided neuroscientists with great tools to study single synapses In Vivo. The H-line and M-line allow facile imaging of major pyramidal cells in the neocortex (Figure 2). However, not all XFP transgenic mice are equally powerful. For example, there are many interneurons that regulate the firing pattern of pyramidal cells[70], but the expression level of XFP is too low in some of these lines[71] to be useful (Crowe and Ellis-Davies, unpublished results). Thus, there is a clear need for more transgenically fluorescent mice to be developed with distinct subsets of neurons brightly labeled by XFP.

Another well-recognized need is a red FP with comparable properties to eGFP. The latter still remains the benchmark after more than 15 years of technology development.[72] It is monomeric, and its fluorescence is bright, highly photostable, and pH insensitive. By comparison, all RFPs are deficient in one or more properties. Tables in refs 72 and 73 provide useful summaries of XFP properties. Why are RFPs considered so potentially useful? First, they form a standard FRET pair with GFP, providing an alternative to the CFP/YFP pair that is normally used. Second, all confocal microscopes are equipped to image green and red. Third, longer wavelength emission offers the possibility of imaging deeper in live tissue. The ultimate goal is to image organs with whole body imagers, and the success of IR dyes such as cy5.5 and cy7.0 have paved the way for genetically encoded IR FP. In this regard, Roger Tsien and co-workers have blazed the trail, with the recent development of two new FPs that emit in the near-IR. Using biliverdin, IFP1.0 and IFP1.4 have a completely different fluorophore from the jellyfish and coral-derived XFPs. However, it seems that the required cofactor bond is not stable in the CNS for more than a few hours. Thus, the "Holy Grail" of a true IR absorption/emission remains elusive.

Finally I would like to highlight the need for genetically encoded sensors of membrane potential as another area that remains wide open for development. Since GECIs are now "coming of age",[33] with transgenic mice being developed,[74] I think it is time to devote more effort to equivalent XYP-based voltage probes. Several attempts have been made,[75] but none are ready for general use.

TWO-PHOTON UNCAGING MICROSCOPY

Studying cell physiology in living animals is important because the cells are in their natural environment: they are truly in situ. However the complexity of the brain severely limits many types of detailed studies and manipulations In Vivo. Thus, neuroscientists often use in vitro preparations to unravel molecular and cellular details. Three forms of cultured preparations are used: (1) acutely prepared brain slices, (2) organotypic brain slice culture, and (3) cells cultured from embryos as monolayers on coverslips. All these in vitro preparations are extremely useful reductionistic approximations of cells In Vivo, with preparations 1 and 2 preserving much of the complexity of brain (but without the long-range connections) and preparation 3 allowing

extremely facile manipulation and access to cells at the expense of removing them from their complex 3D milieu. Two-photon uncaging has been most effectively applied to preparations 1 and 2, because neurons in brain slices faithfully preserve almost all of the electrical and biochemical properties of neurons In Vivo,[76,77] yet it is simple to patch clamp cells in vitro and also perform solution exchange for drug application. Gene gun delivery of DNA is a simple way to label cells in slice culture without the need for transgenics.[78] All areas of the brain can yield brain slices, so DIC optics can be used for visually guided patch clamp. These advantages of brain slices have made them the preparation of choice to study many important problems for synaptic neuroscientists.

Advantages of Two-Photon Uncaging. Even though the exactly the same microscope is used for two-photon fluorescence microscopy and uncaging microscopy, the absence of the confocal pinhole in the former has nothing to do with the advantage of the latter vis-à-vis one-photon (or UV) uncaging. All the advantages of using a two-photon microscope for uncaging are in the excitation path: less light scatter, better depth penetration, and focal excitation. In particular, it is the restriction of excitation in the axial dimension that gives two-photon uncaging a unique advantage over its older, traditional cousin.[5,79] As outlined above, UV−visible light produces the first excited singlet state of any suitable chromophore in the incident path, due to the linear nature of excitation (Figure 1, middle), but nonlinear, two-photon excitation produces excitation in a comparatively small volume,[5,79] hence the punctate appearance of fluorescence emission in Figure 1 (right). The exact dimensions of this volume are defined by the numerical aperture of the microscope lens and the wavelength of light used (the point spread function) and are approximately 0.4−0.6 μm in the x/y directions and 0.9−1.4 μm in the z direction at 720 nm. In order to take full advantage of the point-spread function, photorelease must be faster than diffusion of excited molecules out of the focal volume. The time of exponential decay of excited molecules from the center of the focal volume is about 0.3 ms; thus ideally uncaging should take place in less than 0.1 ms.[79] However this constraint is not absolute for several reasons. First, receptors for the uncaged molecule are not evenly distributed in cells, and responses to uncaging can only be evoked where there are clusters of receptors. Second, there are often uptake systems in cells that powerfully buffer uncaged molecules. Such buffering is most often at the periphery of receptor clusters, so it can appear to constrain uncaging if it is less than ideal in terms of speed. Third, many biological receptors show a nonlinear response to their ligand, so only high concentrations effectively activate responses, and these are only produced at the center of the focal volume. Fourth, some caged compounds are not completely inert biologically, so they can mildly inhibit responses (most commonly observed with caged GABA probes). Since such inhibition is competitive, it will also be more effective at the periphery of the focal volume, because the caged compound is evenly distributed across the sample, but two-photon uncaging is not. With all these caveats for two-photon uncaging, a reader new to the field might reasonably ask the basic question: "But does it work?" In the rest of this review, I will highlight many of the recent striking successes of two-photon uncaging microscopy in the field of chemical neuroscience. All these studies so far have involved using glutamate uncaging to activate AMPA receptors clustered at the tips of dendritic spines.[76,77] Two-photon uncaging is so useful for such studies because (1) the excitation volume is approximately the same size as a large spine and (2) laser energy can be tuned to release a concentration of glutamate that appears to mimic quantal release of a vesicle at such excitatatory synapses (Figure 3). Thus, two-photon uncaging of glutamate at the diffraction limit can be used to "pick off" single dendritic spines in brain slices in a way that closely resembles synaptic stimulation, even when these structures are closely packed together.[80] Since spines are isolated biochemical compartments, local stimulation is required to understand the spatial restrictions that govern their internal chemistry. This is why this method has proven so powerful for synaptic physiology.

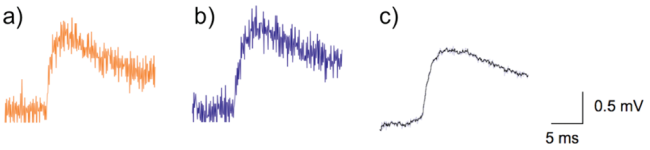

Figure 3. Comparison of photochemically and spontaneously evoked voltage changes recorded from a pyramidal neuron in a living brain slice. (a) Changes in membrane potential evoked by two-photon uncaging of MNI-Glu (bath application at 3 mM) at 720 nm. (b) Example of a single spontaneous miniature excitatory postsynaptic potential (EPSP). (c) Average of many EPSPs. Recordings were made at 23−25 °C from a layer 5 pyramidal neuron in an acutely isolated mouse brain slice.

Development of Caged Glutamate for Two-Photon Photolysis. In 2000−2001, my laboratory developed a caged glutamate designed for two-photon uncaging. Caged calcium probes (DM-nitrophen[81] and DMNPE-4[82−84]) I had made using the dimethoxy-*ortho*-nitrobenzyl chromophore had provided the vital clue as to the starting point for design of a successful two-photon cage for neurotransmitters. Since the electronic-rich dimethoxy chromophores but not the original unadorned nitrobenzyl cage showed decent sensitivity to two-photon excitation, I reasoned that an equivalently caged glutamate probe would undergo effective two-photon photolysis; this proved to be the case.[85] But caged as a benzyl ester, glutamate was easily liberated by hydrolysis before irradiation. This problem was overcome with a monomethoxy nitroindolinyl derivative of glutamate (called MNI-Glu). The preliminary success of MNI-Glu was shown with the Kasai laboratory at the Society for Neuroscience annual meeting in 2000 (abstract 426.12) and published in 2001.[86] In parallel, another group made the same probe[87] but did not think it would be sufficiently sensitive to two-photon excitation for reliable synaptic physiology in brain slices, since their previous work had shown that a very large two-photon cross section (i.e., the ability to absorb photons simultaneously) would be required for such work.[88] However, since our initial publication, many studies have appeared using MNI-Glu for two-photon uncaging. Such work is based on three key properties for the probe:[86] (1) sufficient sensitivity to two-photon excitation for low power uncaging; (2) very fast (submillisecond) synaptic currents evoked by brief (50 μs) flashes of light; (3) MNI-Glu being inert toward AMPA receptors. Note also that MNI-caged transmitters are extremely stable for days at physiological pH at 4 °C.[89]

Diffraction-Limited Two-Photon Uncaging Enables High-Resolution Functional Mapping of Dendritic AMPA Receptor Densities. Studies of receptor distribution in fixed brain slices using high-resolution electron microscopy (EM) had shown that the postsynaptic density (PSD) in spine heads is asymmetrically distributed and that the number of AMPA receptors scales linearly with spine head volume.[90] However

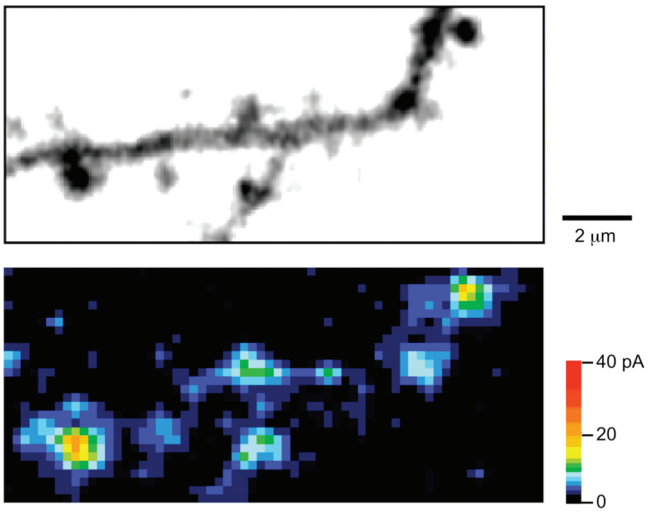

Figure 4. Functional map of AMPA receptor current response from a hippocampal CA1 pyramidal neuron in living brain slice evoked by two-photon uncaging of CDNI-Glu. Upper panel, fluorescent image of a whole cell patch-clamped CA1 neuron filled with Alexa-594 imaged at 950 nm. Lower panel, each pixel was irradiated for 1 ms with mode-locked Ti:sapphire laser at 720 nm and 7 mW, and the evoked postsynaptic current was recorded. The current is shown on a pseudo-color scale. CDNI-Glu was applied from a puffer pipet just above the brain slice at 0.5 mM.

these elegant studies did not show functionality. In contrast, two-photon photolysis of MNI-Glu in living brain slices at the optical diffraction limit revealed that synaptic input into each spine head evoked a current that was directly proportional to spine head volume. Furthermore, it showed that each spine head functioned as its own independent electrical compartment along the dendritic tree.[86] Two-photon mapping was so fine that the asymmetric distribution seen in EM was also readily apparent in the functional map (see Figure 4). It should be mentioned that one of the inventors of two-photon microscopy[6] had previously used their method for functional mapping.[91] This very elegant study laid out all the components of the mapping method from an optical point of view; however since the caged transmitter used was not very sensitive to two-photon excitation, longer irradiation times at high power were required to evoke measurable postsynaptic currents. As a consequence, only low-resolution functional maps of receptor distribution were possible. The success of MNI-caged compounds shows that collaboration between organic chemistry and neuroscience is a powerful way to advance chemical neuroscience.

Biochemistry at Isolated Spine Heads in Brain Slices Using Two-Photon Uncaging of Glutamate. Having established that diffraction-limited two-photon photolysis of MNI-Glu could be used to target single spines, several laboratories used this method for visually guided stimulation of selected spines in living brain slices.[80,92−104] Even though such studies are some of the most refined examples of the use of the uncaging technique, they do no more than apply all the advantages enunciated in the first uncaging experiment by Kaplan et al. in 1978.[105] First, uncaging allows precise, user-defined timing of stimulation of a selected biological process. Second, graded photolysis produces a graded response. If one supposes molecule X produces response Y, graded uncaging allows one to define quantitative relationships between cause and effect in a uniquely powerful way. Third, uncaging bypasses the upstream signaling cascade that produces a particular messenger molecule and any parallel signals that accompany message generation (e.g., DAG with IP_3). The photochemical production of a single messenger molecule can thus dramatically simplify analysis of complex biological signaling systems.

Molecular Control of Spine Head Size. The synaptic basis of memory encoding is thought to involve long-term changes in the connectivity strength of excitatory synapses (called long-term potentiation, or LTP). Since spine head size defines the number of functional AMPA receptors,[90] a logical means of LTP is input-dependent increase in spine size. Two-photon uncaging of MNI-Glu allowed confirmation of this hypothesis in a definitive manner.[30] Since the stimulated spine is visually designated, imaging spine head size before and after a LTP protocol (quantal uncaging[86] at 2 Hz for 1 min at 0 mV) revealed that an increase of spine head volume correlated with an increase in evoked current. A subsequent study proved that this increase in current was due to an increase in the number of AMPA receptors and not channel conductivity.[106] Pharmacological dissection of the signaling cascade confirmed that spine head enlargement required CaM-driven actin polymerization. Several other studies using MNI-Glu have looked in more detail at the molecules involved in spine head enlargement (actin, CaM, CaM kinase).

Using genetically encoded reports of enzymatic activity, Svoboda,[107−109] Yasuda,[99] Oertner,[100] and co-workers have shown that two-photon induction of LTP at isolated spine heads produces local activation of ras and CaM kinase II. The former lasts several minutes and so can spread beyond the spine head compartment and exert an effect as far as 10 μm from the original spine. Nearby spines are then sensitized to "sub-LTP" stimuli so that these spine also undergo structural plasticity.[108] In contrast, CaM kinase II was shown to be activated for a briefer period than ras (ca. 60 s), and in a way, that constrained the new pool of enzyme to the stimulated spine.[99] Thus, the CaM pathway functions as local coincidence detector, whereas the ras pathway is involved in "local chatter" among stimulated synapses.

Neurotrophic factors (e.g., BDNF) control many aspects of protein synthesis and so are well-known to be involved in maintenance of LTP.[110,111] It is now well established that protein synthesis in neurons can take place along the dendritic compartment in addition to the nucleus. Two-photon uncaging of MNI-Glu using a spike-timing-dependent synaptic potentiation protocol was shown to induce a protein-synthesis-dependent enlargement in spine head size[112]. Since uncaging only affected the visually targeted spine and not nearby spines, the photochemically controlled synaptic input must produce a molecular tag that localizes protein synthesis to the stimulated spine. Use of a BDNF antibody proved that autocrine secretion of the neurotrophic factor was responsible for the protein-dependent part of spine head enlargement.

Calcium Signaling at Single Spine Heads. The essential role of calcium in LTP is established; entry of calcium through NMDA receptors is required for potentiation.[113] Two-photon uncaging of MNI-Glu has allowed many elegant studies of this process in a highly controlled manner. Filling neurons with a fluorescent calcium dye by whole-cell patch clamp technique allows direct visualization of calcium fluctuations in single spines.[114] When regular synaptic stimulation is used, it is only by chance that one can "catch" the individual spine head changes in Ca^{2+} concentration; two-photon uncaging allows one to bypass this laborious process, since the experimenter can simply

Table 1. Summary of the Properties of the Widely Used or Recently Developed Caged Glu Probes[a]

caged Glu	ε (λ_{max})	ϕ (%Glu yield)	$\varepsilon\phi$	2P evoked voltage |rise time (ms)	pharmacology toward GABA-A	stability in aqueous buffer	solubility (mM) at pH 7.4 in aqueous buffer
CNB[137]	500 (350)	0.14 (100)	60	no 2P current	partial agonist	half-life 17 h RT	>50
MNI[86,87]	4300 (330)	0.085 (>95)	357	3.5	strong antagonist at 10 mM	stable	1400
RuBi[135]	5600 (450)	0.13 (NQ)	728	14	50% inhibition at 0.3 mM	stable	NR
PMNB[138]	9900 (317)	0.1 (100)	990	NR	NR	stable	requires 1% DMSO
antMNI[134]	27000 (300)	0.085 (94)	2295	NR	NR	stable	33
BNSF[133]	64000 (415)	0.25 (65)	16000	NR	NR	NR	0.1
CDNI[130]	6400 (330)	0.6 (100)	3840	NR	mild antagonist at 0.4 mM	stable pH 2	100

[a] Abbreviations and symbols: 2P, two-photon; NQ, not quantified but probably high; NR, not reported; ε, extinction coefficient (M^{-1} cm^{-1}); λ_{max}, wavelength maximum of absorption (nm); ϕ, quantum yield of photolysis.

select spines visually for study.[86] The quantitative details of the calcium signaling process when studied with two-photon photolysis of MNI-Glu have been a matter of some controversy.[76,77,115] Some laboratories suggest that as much as 99% of spine head calcium is buffered within the spine,[94,116−120] whereas others have detected significant calcium signals entering the adjacent dendrite, especially through wide and short spine necks connected to large spines.[121] Why has this range of findings been reported? Disparate viewpoints may arise because each laboratory uses slightly different conditions to study the calcium signaling process. A range of calcium dyes at different concentrations have been used; thus the degree of artificial calcium buffer capacity varies over more than an order of magnitude. (For an excellent introduction to this see ref 122.) Further, the temperature of the brain slice medium also varies over a significant range[94,116−118,121] (25−36 °C). Since endogenous calcium buffering is significantly changed by temperature, this experimental difference has not helped reconcile differences. The exact mode of calcium imaging also differs among various studies. Some microscopes are equipped with two independently controlled $x-y$ mirrors that permit scan rotation of the image frame with precise uncaging,[98,103,121,123] while others capture small frames at a slightly slower rate, but with more spatial information.[94,99,107,124] Finally, the amount of photochemical stimulation used has varied by approximately a factor of 3. A combination of these different experimental parameters probably explain the divergence of opinion among various groups.

What are the major physiological functions of spine head calcium, and how does calcium-buffering dynamics affect such functions? All laboratories would agree that small spines with long spine necks retain all calcium entering the isolated compartment of the spine head. If sufficient calcium enters through NMDA receptors in a temporally restricted time, then LTP at that spine on a pyramidal neuron takes place, along with concomitant spine head enlargement.[30,99,100,121] The composition of the NMDA-R subunits has been studied in detail using two-photon uncaging.[94,117] Since spines also possess voltage-gated ion channels, synaptic input onto a spine can also activate calcium-permeable ion channels indirectly, further enhancing the increase in Ca^{2+} concentration due to NMDA receptors.[115] Two-photon uncaging of MNI-Glu has been used to study these channels in exquisite detail. In particular, Sabatini and co-workers have studied the modulation of Ca(v2.3) on spine heads.[96,116,118,119,125] They have found that this voltage-gated ion channel is intimately linked in space to yet another channel on spine heads, the small conductance Ca^{2+}-activated potassium (or SK) ion channel. They found that increases in Ca^{2+} concentration in spines increase LTP via NMDA receptors but also inhibit LTP via Ca(v2.3)/SK, giving rise to apparently conflicting functions of spine Ca^{2+}. Sabatini and co-workers concluded that the latter are in a privileged microdomain where large (ca. 10−20 μM), highly local increases in Ca^{2+} via Ca(v2.3) activate adjacent SK channels and the SK channels hyperpolarize spine head membrane potential, thus inhibiting LTP. In contrast, the changes in spine head Ca^{2+} from NMDA receptors is only 1−2 μM, so it does not rise to sufficiently high levels to stimulate SK channels. Thus depending on its origin, spine Ca^{2+} can have bidirectional effects on LTP.

Using Two-Photon Uncaging of Glutamate To Study Dendritic Branch Conduction Properties. The preceding discussion concerned the physiology of single spine heads. Two-photon uncaging of glutamate is ideally suited for such studies because it enables rational stimulation of visually designated spines in a facile way. In principle, minimal synaptic stimulation could be used for much of this work, but two-photon uncaging is "easier" because of its direct connection of cause and effect. Recently some more elaborate studies have appeared exploiting a capability that is unique to two-photon uncaging, namely, rationally targeted patterned uncaging at multiple spine heads. Such work is a logical extension of functional mapping,[86] in that some two-photon microscopes allow an arbitrary number of uncaging events to be defined in space and time. Thus, on pyramidal neurons, uncaging at several closely spaced spine heads in rapid succession produced nonlinear summation of the expected electrical signal along a stimulated dendritic branch.[123,126] Such "patterned input" is only possible with uncaging, because stimulation of axonal afferents has some inherent arbitrariness due to the stochastic nature of their response and random nature of their projection pattern. Even more refined studies using multisite uncaging of MNI-Glu have been used to study the biophysical properties of branch segments of CA1 pyramidal neurons in acute brain slices.[103,127]

Using Two-Photon Uncaging of Glutamate to Fire Action Potentials. The raison d'être of neurons is to fire action potentials. Some of the earliest applications of UV uncaging of glutamate were to fire action potentials in brain slices, to produce broad scaled maps of functional neuronal connectivities.[128,129] Surprisingly two-photon uncaging of glutamate has been used in few studies to fire neuronal spikes, probably because the normal two-photon excitation does not release enough glutamate to initiate a spike easily. Proof of principle of the ability of two-photon uncaging of MNI-Glu to do this came first from Yuste and co-workers who developed a clever means of distributing the uncaging beam onto the cell body of neurons in brain slices.[95]

With use of a dispersive element to multiplex the uncaging beam into several spots that hit the cell simultaneously, sufficient glutamate could be released onto one cell to fire a spike. Shortly after this report, Matsuzaki and co-workers increased the two-photon uncaging volume by underfilling the back aperture of the microscope objective to uncage CDNI-Glu[130] on neurons in acutely isolated brain slices.[131] This new method enabled mapping of long-range connectivity patterns of pyramidal neurons in the neocortex with high resolution. Finally, in the first example of two color, two-photon uncaging of two neurotransmitters, action potentials were fired using multiple-spine head uncaging of CDNI-Glu at short wavelengths (720 nm). Such spikes could be blocked by independently uncaging of GABA at longer wavelengths (830 nm) around the soma.[132]

Beyond MNI-Glu, Development of New Caged Transmitters for Two-Photon Photolysis. Since MNI-Glu was developed for two-photon photolysis in 2001,[86] many other laboratories have independently reported its effectiveness for stimulation of single heads (vide supra). However, this caged compound is not "perfect", so many chemists have tried to develop probes with improved properties, to make two-photon uncaging experiments even more useful. Three approaches have been taken: (1) improve nitroindolinyl photochemistry, (2) use alternative organic chromophores with larger two-photon cross sections or (3) develop hybrid organic−inorganic cages with the hope of improved chemistry. Table 1 lists the properties of the best of these new caged glutamate probes.

It can be seen that BNSF-Glu has easily the highest two-photon cross section,[133] seemingly making it a very attractive probe for study of synaptic signaling in brain slices. However, it is sparingly soluble (0.1 mM), and this probably makes brain slice experiments tricky to perform on a day-to-day basis. Addition of highly charged phosphate moieties to caging chromophores should provide a simple solution to this quandary. However attempts to use this approach have shown that the juxtaposition of bisphosphates and condensed aromatic systems causes precipitation of probes in physiological buffer.[134] Hybrid organic−inorganic caging chromophores (bipyridyl-rutheniums) are soluble in physiological buffer.[135] However two-photon uncaging of such probes at single spine heads was reported to produce comparatively slow rise times in voltage clamp (4 ms), even though spine stimulation in the axial dimension was better than the diffraction limit (<0.9 μm). Two approaches to improving MNI-Glu have been advanced. The first attempts to improve the chromophore cross section by appending an antenna that initially absorbs the light and then transfers excitation to the MNI moiety.[134] A modest 2-fold improvement in efficiency of release at 350 nm was reported (Table 1). Finally, addition of a second nitro group to MNI improves the efficiency of release ca. 6-fold.[130] This significant improvement in efficacy allows much lower concentrations (0.5−2 vs 10 mM) of caged Glu to be applied to brain slices, while still allowing effective two-photon stimulation (Figure 4). Thus, CDNI-caged glutamate and GABA[136] seem, on balance, to be the best caged transmitters to date, in terms of chemistry, photochemistry, solubility, and pharmacology.

Future Challenges for Caged Compound Development. The main challenge in the development of caged neurotransmitters seems to be blockade of GABA-A receptors. Remarkably, all caged transmitters seem to bind to these receptors at the concentrations required for two-photon uncaging. Highly water-soluble caged transmitters with large two-photon cross sections would allow bath application of probes to brain slices at subinhibitory concentrations so as to allow detailed study of synaptic integration in brain slices or uncaging in living animals. These applications are, in my view, the two "final frontiers" of the two-photon uncaging technique.

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