Published in final edited form as:

J Microsc. 2007 April ; 226(Pt 1): 74–81.

Limited Utility of Acetoxymethyl (AM) Based Intracellular Delivery Systems, *in vivo*: Interference by Extracellular Esterases.

Paul D. Jobsis[#], Emily C. Rothstein⁺, and Robert S. Balaban^{*}

Laboratory of Cardiac Energetics, National Heart Lung and Blood Institute, Bethesda MD, 20892

Abstract

The use of acetoxymethyl(AM) groups to deliver and trap exogenous optical probes inside cells is an established tool in cell biology/physiology, however, these probes have not been used extensively *in vivo*. In this study, the use of the acetoxymethyl delivery system for optical probes was evaluated, *in vivo*. Initial studies revealed very little trapped probe in intact tissues even when near saturating levels of probe were injected in living animals. We tested the hypothesis that extracellular esterases rapidly cleave the AM groups preventing the probes from entering cells, *in vivo*. The rates of hydrolysis of 11 AM probes in diluted porcine plasma revealed an essentially first order high rate dye cleavage with half times on the order of minutes or less. Studies on mice and rabbits revealed rates 10 to 2 fold higher respectively. These plasma studies suggested that the AM probes were being cleaved before having a chance to enter cells in tissues *in vivo*. This was confirmed using intra-vital 2-photon excitation microscopy in muscle tissue where several AM probes were found to rapidly cleave in the vascular space during infusion and not be trapped in the muscle cells. Studies with succinimidyl esters that should quickly bind to proteins on cleavage also failed to enter cells, *in vivo*, consistent with the notion that the cleavage was occurring in the extracellular space. These data suggest that the high level of plasma and extracellular esterase activity render the classical AM probes ineffective for monitoring intracellular events, *in vivo*. Different approaches to trapping exogenous probes will need to be explored for physiological studies using intra-vital microscopy.

Keywords

Mouse; skeletal muscle; SNARF; extracellular space; two-photon; microscopy; INDO; rabbit; spectroscopy; vascular imaging; nuclei

INTRODUCTION:

The use of ester based chemistry to deliver probes into the cytosol was based on the classical work of Tsien (23). This approach works by screening charged sites with acetoxymethyl (AM), acetate or other ester groups creating a hydrophobic molecule that can enter cells across the plasma membrane by simple diffusion. Once inside the cell, cytosolic esterases cleave the acetoxymethyl groups revealing the charged sites trapping the now hydrophilic molecule in the cytosol. Often the unveiling of these charged sites also generates some of the functional groups of the probe for ion detection and/or results in fluorescence enhancement to selectively label the cytosol. This approach has essentially revolutionized cell biology permitting the non-invasive imaging of the cellular milieu including pH(2;24), Ca²⁺(25), Mg²⁺(17) as well as simply providing a better illumination of the cellular morphology. This approach has proven

^{*}Corresponding author: Robert S. Balaban, Laboratory of Cardiac Energetics, National Heart Lung and Blood Institute, Bldg 10, Room B1D161, Bethesda, MD 20897. Email rsb@nih.gov Telephone: 301 496-3658

[#]Current address: University of the Virgin Islands, Department of Science and Math, #2 Brewers Bay Rd, St Thomas USVI 00802

⁺Current address: Eli Lilly, Indianapolis, IND

to be useful on primary cells *in vitro* or cells in culture as well as isolated perfused organs(5; 13;20;26) These studies have led to many new important insights into the function of cells and organs under normal and patho-physiological situations, *in vitro*. Logically to be able to measure these parameters *in vivo* (i.e. in the living animal) would provide critical information on the innate function and regulation of many important biological processes in their most natural state. Despite the development of methods of conducting cellular observations *in vivo* (see (3)), very few reports of successful use of this class of probes *in vivo* have been reported. Recent surveys of useful probes for *in vivo* microscopy notably do not include AM based dyes(4). Stosiek et al (21) reported that the direct pressure injection of 1mM AM dye in the region of super-fused brain is effective in labeling neurons. In our own laboratory we attempted to use a variety of these probes *in vivo* for several applications with little or no success using mostly vascular delivery approaches. This included vascular infusions in mice and rats for several hours exceeding the concentrations used in isolated organ perfusion studies in the same animals, local infusions of dyes into the coronary vasculature using local infusion techniques(12) as well as injecting dyes directly into the pericardial fluid at extremely high concentrations for hours.

These observations led to two hypotheses with regard to why the AM based probes were not effective, *in vivo*. The first hypothesis was that the probes were being cleaved by plasma or interstitial esterases. The second hypothesis was the organic acid export systems were more effective, *in vivo*, driving the cleaved dye out of the cell.

The purpose of this study was to characterize the extracellular esterase activity in mammalian systems to estimate the lifetime of the uncleaved probes in the vascular space to test the first hypothesis. The second hypothesis was tested *in vivo* using probes with the amide reactive group, succinimidyl ester, that would remain in the cytosol by attaching to protein structures (14) after cleavage and not be exported by organic acid export mechanisms. The results of these studies were consistent with the first hypothesis, that is; the esterase activity in the extracellular space dominating the handling of these probes, *in vivo*.

Materials and Methods

In Vitro Plasma Experiments

Measurement of plasma cleavage rates were performed using porcine plasma at 37 ± 2 C in a temperature controlled spectro-fluorometer (J3, Jobin Yvon). In preliminary studies, we found that the rate of cleavage was dependent on plasma dilution with the highest rates reached with 100% plasma, however, plasma absorbance of the emission and excitation light (secondary and primary inner filters) made quantitative studies difficult. Thus, we used the 1/3 dilution and assumed that the 100% plasma values could be linearly extrapolated. A one third dilution of plasma with 10mM Hepes buffer (pH=7.4 at 37°C) was used unless specified differently. The half lives of 11 different fluorescent probes or indicators were determined by simply monitoring the appropriate fluorescence emission as a function of time. The concentration appropriate to obtain a large fluorescent signal was found for each probe and used throughout testing of that probe. For one probe, 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (carboxy-DCFDA), esterase activity at concentration covering three orders of magnitude was determined. Concentrations of 0.05, 0.5 and 5.0 μ M were used. Higher concentrations of probes were not used due to self quenching. For all calcium indicators 2mM of CaCl_2 was added prior to addition of Ca^{2+} indicator to uniformly use the fully bound form of the dye. Separate measurements of carboxy-DCFDA were made at 25 and 39 degrees to determine the temperature dependence of the plasma esterase activity.

Interspecies comparison of plasma esterase activity was made using plasma samples from 5 male mice, two male rabbits and three pigs. Interspecies comparison was made using carboxy-

DCFDA at room temperature (25°C) using a 10 and 20x dilution with Hepes buffer (10mM, pH 7.4@25°C) of the plasma to obtain esterase concentrations that allowed accurate interspecies comparisons.

***In vivo*, Experiments**

Fifteen male mice were anesthetized with isoflurane prior to tail vein catheterization and surgical exposure of skeletal muscles in the lower back and right rear leg including the longissimus dorsi and gluteus medius. The serous membranes covering the muscle were carefully removed to avoid bleeding and damage to the muscle fibers. The mouse was kept warm and arranged on a Zeiss LSM 510 NLO inverted microscope stage using a 5mm plexiglass plate with a 2x3 cm opening sealed with a large cover-glass. The skeletal muscles were imaged using two-photon induced auto-fluorescence (NAD(P)H) with either a Zeiss 20x or 40x lens. Emission data was collected using multiple photomultiplier tubes appropriately filtered for NAD(P)H or the probe emission under study. In some cases full spectral information was collected using the resident META spectroscopy system within the LSM 510 system with appropriate spectral correction(10). When an appropriate field of view was found the AM fluorescent probe was infused with either a bolus or a constant infusion rate to reach an estimated plasma dye loading concentration between 50 and 100 μ M. Several probes were evaluated including carboxy-DCFDA, 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Invitrogen # C-1157), 5-(and -6)-carboxy, SNARF - succinimidyl ester (SNARF-S, Invitrogen #C-1271), and 5-(and -6)-carboxy SNARF (SNARF, Invitrogen #C-1270).

A second *in vivo* preparation was the rabbit tibialis anterior (TA) muscle using a preparation previously reported (19). The rabbit was used since the larger vessels permitted a direct infusion of dye into the arterial system of the lower leg via the iliac artery. In these studies, a 4 F catheter was placed in the iliac artery using an introducer in the carotid artery under fluoroscopic guidance. The field of perfusion was confirmed by pulsing radio-opaque dye down the catheter during the fluoroscopic exams. This arrangement permitted the minimally invasive direct infusion of the dye into the arterial system avoiding the full circulation required with the venous injections in the mouse, minimizing the time exposed to extracellular esterases. A 20mM DMSO stock solution of dye was infused in the artery at a rate to generate a $\sim$ 100 μ M concentration assuming a blood flow rate of 10 ml/min. The total injection was 2 mls of dye solution delivered over $\sim$ 16 minutes ($\sim$ 0.05 ml/min). Images were collected over the entire infusion time as well as for several minutes after the infusion.

The dye distribution through the tissue was determined using a linear least squares fitting routine to extract the energy from overlapping emission spectra (6). In these studies the background fluorescence, likely from NAD(P)H, was used to identify the center of a cell as well as provide a marker of the cell. A model background spectrum (M_{BS}) was taken before the infusion of dye. The model dye emission spectrum (M_{DS}) was collected from either the vascular space or clearly defined cellular interspaces. It is important to note that these spectra were collected as close to the actual region of observation of the experimental measurements to minimize any differential effects of inner filters on the spectral properties (18). The integrated areas of M_{DS} and M_{BS} were normalized to the same value to permit the direct comparison of the relative contribution of these two spectra to the observed experimental spectra. To estimate the level of dye within a cell, spectra (CS) were collected in the middle of the cell as determined from a z-stack of the tissue using the same excitation parameters used to collect M_{BS} and M_{DS} . The CS spectrum was then fitted using BS and DS using the following equations:

$$1. \quad CS = \text{Background Fluorescence} + \text{Dye Fluorescence}$$

$$2. \text{ Background Fluorescence} = I_{BF} + M_{BS}$$

$$3. \text{ Dye Fluorescence} = I_{DF} + M_{DS}$$

The coefficients I_{BF} , and I_{DF} were determined in the spectral fitting program to estimate the contribution of each component to the spectrum. *Equation 1* was used to fit the observed cellular fluorescence spectrum after dye infusion using the linear least-squares algorithm previously described(9). Data is generally reported as I_{DF}/I_{BF} to estimate the level of dye enhancement in the cell.

Results

Plasma Esterase Activity

The rate of acetoxymethyl - probe hydrolysis was much faster in diluted plasma than in buffer alone (Table 1). All probes tested showed a half life in the Hepes buffer of greater than one hour. However in the diluted porcine plasma (one third Hepes buffer two thirds plasma) there was a very robust cleavage of the probes with half lives ranging from 10 minutes to less than one minute. In comparative studies, esterase activity on carboxy-DCFDA was greater in mouse (89.5 nM/ml/sec) and rabbit (14.9 nM/ml/sec) plasma when compared to pig plasma (7.0 nM/ml/sec).

Evaluation of substrate dependence on enzyme activity of carboxy-DCFDA at concentrations 50 nM, 500 nM and 5 μ M surprisingly did not show evidence of substrate saturation, even with these diluted plasma samples, with the half lives in seconds being 57 ± 4 , 57 ± 11 , 56 ± 10 respectively. These data suggest that the capacity of the esterase is remarkably high in the plasma of even the pig and that it can not be overcome with high concentrations of AM dye.

The plasma esterase cleavage of carboxy-DCFDA was 0.7%/sec at 25°C and 1.7%/sec at 39°C. This data corresponds to a Q_{10} of 2.1 consistent with most enzymatic reactions. These data also suggests that temperature will not be a powerful modulator of this process.

In vivo Studies

In general, all of the *in vivo* infusion schemes did not result in significant cellular loading of fluorescent probe. An example of the mouse systemic infusion of carboxy-DCFDA is shown in time course illustrated in Figure 1. The images shown were collected at zero, 1, 2 and 10 minutes after infusion. All of the fluorescence was limited to the extracellular space using the NAD(P)H fluorescence at ~450 nm as a marker of intracellular space. The qualitative time course of dye fluorescence channel intensity is presented in Figure 2. These data are from small regions of interest located in a capillary, cell and extra-cellular space. The rise in signal occurred first in the vascular space followed by a near linear increase in the extra-cellular space. The cellular signal was low throughout the time course. Quantitative spectral analysis confirmed these qualitative measures with the I_{DF}/I_{BF} being less than 0.1 in cellular regions of interest in spectral analysis collected at 10 minutes after the infusion. Waiting beyond 20 minutes reduced the extracellular signal but did not increase cellular loading. Similar results were obtained with injections of SNARF.

To test the hypothesis that the cleaved probes were present in the cellular compartment and were exported out of the cell by the organic acid transport systems, such as the ABC transporter (8), we used the amine reactive succinimidyl linked probes that should be trapped in the cytosol after being cleaved by esterases. The analysis of a single time point 10 minutes after a systemic CFDA-SE injection in the mouse is shown in Figure 3. The spectra (M_{DS} and M_{BS}) used to segment the NAD(P)H and CFDA-SE signals are presented in the panel marked spectra. Again, the probe was only located in the extracellular space labeling capillaries and intercellular spaces with the I_{DF}/I_{BF} remaining under 0.1. Similar results were obtained with SNARF-S (not

shown). Motion artifacts from breathing are indicated by the asterisks(19), and are not physical structures. The fact that the succinimidyl probes were not retained in the cells, suggest that the export of the cleaved probe is not the problem, but that the probe never gets the chance to enter the cells, *in vivo*.

In an attempt to minimize the time the probe was exposed to the plasma esterases and maximize the concentration of probe being delivered prior to reaching the tissue of interest, a direct arterial injection of the probe was used in rabbits. A representative example of these studies is presented in Figure 4 for CFDA-SE into the iliac artery while monitoring the TA is presented. Again, the probe was limited to the extra-cellular space including veins (v) in this series. Similar results were obtained in 3 animals. In addition, SNARF-S was injected into 2 animals with similar results. Thus, even the direct arterial infusion of the probes was not adequate to overcome the extracellular esterase activity.

Discussion

These data support the notion that the extracellular esterase activity cleaves AM probes preventing adequate delivery of the permeant form to enter most cells, *in vivo*. This conclusion was supported by the demonstration of a rapid essentially first order cleavage of AM dyes in the plasma. In addition, dye cleavage was observed in the blood and interstitial regions *in vivo* using intravital microscopy, with little or no labeling of intracellular compartments consistent with the rapid cleavage of dye in the extracellular space. Further evidence that the cleaved dye was never present in the cytosol was the inability of an added amine reactive group to the probes to trap the molecules within cells.

The esterase activity measured in plasma, *in vitro*, is likely an underestimation of the true activity in whole blood due the absence of erythrocyte esterase activity(16). The plasma has many esterase enzymes and it is likely that more than one is responsible for cleavage of the ester bonds in AM probes(1). The near first order kinetics of AM dye cleavage suggests that the esterase activity is very high, and can not be saturated using excessive concentrations of dye. This potentially explains why even very high levels of infused AM dyes did not result in skeletal muscle cellular loading. Similar results were observed in the mouse kidney and liver in these protocols. Similar results were found in the rat kidney, *in vivo*, with the AM probes (KW Dunn, personnel communication). The Q_{10} (~ 2) of the plasma esterase reaction was also not remarkable and does not suggest that modifying temperature would be an effective mechanism of overcoming the esterase activity.

In vivo the cleavage of the dye could be observed almost immediately by observing the vascular space just below the injection site. The probes clearly distributed throughout the extracellular space with little or no dye being present in the muscle cells. In some cases dye was apparently loaded in the endothelial cell of the vasculature, consistent with previous studies on perfused organs(15). The time course of the cleavage within the vascular spaces is difficult to determine since it is difficult to separate vascular delivery from cleavage within the living animal. However, a time constant on the order of seconds is consistent with the rapid hydrolysis of probe observed, *in vivo*. Adding the probe, at near saturating concentrations, to the surface of the *in vivo* muscle in these studies, and previous studies on the porcine and canine heart, also revealed no apparent muscle cell labeling, consistent with the notion that the extracellular space in these tissues is rich in esterase activity.

The possibility remained that the probes were entering cells, cleaved, and then vigorously exported by export systems, such as ABC transporters(8), resulting in the elimination of the probe from the cytosol. To test this hypothesis, probes with the amine reactive group, succinimidyl ester form, were used with the notion that if cleaved probe was present in the

cytosol, then it would be trapped by its interaction with proteins. All of the succinimidyl derivatives did not improve the cellular loading but did make the labeling of the extracellular space more persistent, consistent with the amine reaction. Thus, these data suggest that cleaved AM dye was never present in the cytosol and that dye cleavage was occurred in the cellular interspaces and not just in the plasma. The rapid hydrolysis of the AM dyes in the extracellular space, and not extrusion of the cleaved dye, also explains why the AM probes can easily be loaded into perfused organs or isolated cells with no difficulty where the plasma, and the extracellular space proteins, have been washed out.

There are several methods that might be use to reduce extracellular esterase activity to load these useful AM dyes, *in vivo*. Stosiek et al (21) may have been successful in loading AM dyes in the brain due the dilution of the extracellular esterases by the superfusion of the brain. Another possibility is that the blood brain barrier prevented the plasma esterases to enter the brain extracellular space resulting in a lower esterase activity in the brain intercellular spaces. Likely, the superfusion together with the isolation of the intercellular spaces from the plasma by the blood-brain barrier contributed to the successful loading of AM dyes in this preparation. Similar approaches of transiently perfusing an organ system with a Ringer's solution and dye, *in situ*, might overcome this effect much like perfused organs *in vitro*. The possibility of using infusions of esterase inhibitors such as Diisopropyl fluorophosphate (Sigma D0879), Bis-p-nitrophenylphosphate (BNPP), Eserine (Sigma E8375), 5,5'-dithiobis-(2-nitrobenzoic acid) (Sigma D8130) or p-chloromercuribenzoate (Sigma H0642), to allow the AM probes to reach the target organ and cells may also be a reasonable strategy if these agents stay in the extracellular space. But the serious side affects of these inhibitors, some of which have been used as chemical weapons, would again raise doubts as to applicability of the resulting measurements to normal physiological function. In preliminary studies in isolated plasma samples we found significant inhibition of the esterase activity only at very high (mM) concentrations of inhibitors suggesting that toxicity would be a significant problem since many esterases are required for normal physiological function. Finally, it is unclear that modifying the probes themselves to non-selectively protecting the acetoxymethyl, or similar group, to increase plasma lifetime would improve the selective delivery to the cytosol. A longer lifetime, even permitting equilibrium of the uncleaved probe between the pools, will only result in selective cytosolic labeling if the cytosolic esterase activity far exceeds the extracellular space. Based on the high extracellular esterase activity observed in this study, it is unlikely that simply increasing the lifetime of the uncleaved AM-probes would be of much value in selectively labeling the cytosol. However, the esterase specificity in the extra and intracellular space may be sufficiently different to adjust the AM probes to be more resistant to the extracellular esterase activity. This would permit selective trapping of a probe in the cytosol. To our knowledge, no information on the specificity of the extracellular and intracellular esterases is available.

It is likely that different methodology will be needed to get these types of valuable probes into the cytosol of cells, *in vivo*. We have found that most lipophilic dyes such as most of the Syto dyes (Invitrogen) for nuclei labeling, ANEPPS-8 or -4 (Invitrogen) and Acridine Orange were very effective at labeling structures *in vivo* (Figure 5), consistent with the survey of probes evaluated by Dunn et al (4). The cellular labeling of DAPI ((4) and the Syto dyes (Figure 5) demonstrate that these class of probes can easily across the capillaries and extracellular space deep into tissues, *in vivo*. These results suggest that these classes of probes would make good initial model compounds for future probe development for *in vivo* studies. Other chemical approaches may need to be employed including protease activated probes(7), genetically encoded ion sensitive fluorescent proteins(22) or di-sulfur bound probes(11) that use different strategies to trap molecules in the cytosol.

In summary, we have shown that it is exceedingly difficult to load AM dyes into cells, *in vivo*, via the vasculature. This is apparently due to the high activity of esterases in the plasma

and extravascular space. Even very high concentrations of dye directly infused into the arterial system will not overcome the essentially first order reaction in the plasma. These results suggest that the classical AM dyes will have limited utility, *in vivo*, and that new approaches to exogenous dye delivery need to be explored.

Acknowledgements:

The authors would like to thank Mr. Jamie Schroeder and Dr. Merav Luger-Hammer for the Acridine Orange stained images of the *in vivo* mouse. Dr. KW Dunn for some very useful discussions on the application of AM probes in the mammalian kidney. Finally, we would like to acknowledge the previous workers in the Laboratory that contributed to the development of this project including Drs. Andrew Arai, Timothy Ryschon, and Paul Territo.

Reference List

1. AUGUSTINSSON KB. Electrophoretic separation and classification of blood plasma esterases. *Nature* 1958;181:1786–1789. [PubMed: 13566141]
2. Bright GR, Whitaker JE, Haugland RP, Taylor DL. Heterogeneity of the changes in cytoplasmic pH upon serum stimulation of quiescent fibroblasts. *J Cell Physiol* 1989;141:410–419. [PubMed: 2478571]
3. Cahalan MD, Parker I, Wei SH, Miller MJ. Two-photon tissue imaging: seeing the immune system in a fresh light. *Nat Rev Immunol* 2002;2:872–880. [PubMed: 12415310]
4. Dunn KW, Sandoval RM, Kelly KJ, Dagher PC, Tanner GA, Atkinson SJ, Bacallao RL, Molitoris BA. Functional studies of the kidney of living animals using multicolor two-photon microscopy. *Am J Physiol Cell Physiol* 2002;283:C905–C916. [PubMed: 12176747]
5. Fralix TA, Heineman FW, Balaban RS. Effects of tissue absorbance on NAD(P)H and INDO-1 fluorescence from perfused rabbit hearts. *FEBS Lett* 1990;262:287–292. [PubMed: 2335209]
6. French SA, Territo PR, Balaban RS. Correction for inner filter effects in turbid samples: fluorescence assays of mitochondrial NADH. *Am J Physiol* 1998;275:C900–C909. [PubMed: 9730975]
7. Funovics M, Weissleder R, Tung CH. Protease sensors for bioimaging. *Anal Bioanal Chem* 2003;377:956–963. [PubMed: 12955390]
8. Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* 1993;62:385–427. [PubMed: 8102521]
9. Heineman FW, Eng J, Berkowitz BA, Balaban RS. NMR spectral analysis of kinetic data using natural lineshapes. *Magn Reson Med* 1990;13:490–497. [PubMed: 2325549]
10. Jobsis PD, Combs CA, Balaban RS. Two-photon excitation fluorescence pH detection using 2,3-dicyanohydroquinone: a spectral ratiometric approach. *J Microsc* 2005;217:260–264. [PubMed: 15725129]
11. Jones LR, Goun EA, Shinde R, Rothbard JB, Contag CH, Wender PA. Releasable luciferin-transporter conjugates: tools for the real-time analysis of cellular uptake and release. *J Am Chem Soc* 2006;128:6526–6527. [PubMed: 16704230]
12. Laughlin MR, Taylor JF, Chesnick AS, DeGroot M, Balaban RS. Pyruvate and lactate metabolism in the *in vivo* dog heart. *Am J Physiol* 1993;264:H2068–H2079. [PubMed: 8322935]
13. Lee H-C, Smith N, Mohabir R, Clusin WT. Cytosolic calcium transients from the beating mammalian heart. *Proc Natl Acad Sci* 1987;84:7793–7797. [PubMed: 3478728]
14. Lomant AJ, Fairbanks G. Chemical Probes of Extended Biological Structures - Synthesis and Properties of Cleavable Protein Cross-Linking Reagent [Dithiobis(Succinimidyl-S-35 Propionate)]. *Journal of Molecular Biology* 1976;104:243–261. [PubMed: 957432]
15. Lorell BH, Apstein CS, Cunningham MJ, Schoen FJ, Weinberg EO, Peeters GA, Barry WH. Contribution of endothelial cells to calcium-dependent fluorescence transients in rabbit hearts loaded with indo 1. *Circ Res* 1990;67:415–425. [PubMed: 2376080]
16. Pelzer CF. Genetic control of erythrocytic esterase forms in *Mus musculus*. *Genetics* 1965;52:819–828. [PubMed: 5826325]
17. Raju B, Murphy E, Levy LA, Hall RD, London RE. A fluorescent indicator for measuring cytosolic free magnesium. *Am J Physiol* 1989;256:C540–C548. [PubMed: 2923192]

18. Rothstein EC, Carroll S, Combs CA, Jobsis PD, Balaban RS. Skeletal muscle NAD(P)H two-photon fluorescence microscopy in vivo: topology and optical inner filters. *Biophys J* 2005;88:2165–2176. [PubMed: 15596503]
19. Rothstein EC, Nauman M, Chesnick S, Balaban RS. Multi-photon excitation microscopy in intact animals. *J Microsc* 2006;222:58–64. [PubMed: 16734715]
20. Seo JT, Steward MC, Larcombe-McDouall JB, Cook LJ, Case RM. Continuous fluorometric measurement of intracellular pH and Ca²⁺ in perfused salivary gland and pancreas. *Pflugers Arch* 1994;426:75–82. [PubMed: 8146028]
21. Stosiek C, Garaschuk O, Holthoff K, Konnerth A. In vivo two-photon calcium imaging of neuronal networks. *Proc Natl Acad Sci U S A* 2003;100:7319–7324. [PubMed: 12777621]
22. Tallini YN, Ohkura M, Choi BR, Ji G, Imoto K, Doran R, Lee J, Plan P, Wilson J, Xin HB, Sanbe A, Gulick J, Mathai J, Robbins J, Salama G, Nakai J, Kotlikoff MI. Imaging cellular signals in the heart in vivo: Cardiac expression of the high-signal Ca²⁺ indicator GCaMP2. *Proc Natl Acad Sci U S A* 2006;103:4753–4758. [PubMed: 16537386]
23. Tsien RY. A non-disruptive technique for loading calcium buffers and indicators into cells. *Nature* 1981;290:527–528. [PubMed: 7219539]
24. Tsien RY. Fluorescent indicators of ion concentrations. *Methods Cell Biol* 1989;30:127–156. [PubMed: 2538708]
25. Tsien RY, Pozzan T, Rink TJ. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. *J Cell Biol* 1982;94:325–334. [PubMed: 6980885]
26. Wurzinger R, Englisch R, Roka S, Langer R, Roden M, Graf J. Intracellular calcium in the isolated rat liver: correlation to glucose release, K(+) balance and bile flow. *Cell Calcium* 2001;30:403–412. [PubMed: 11728135]

Figure 1.

In vivo mouse skeletal muscle fibers imaged by two-photon (710nm excitation) induced fluorescence (A) before (B), one minute (C) two minutes and (D) 20 minutes after infusion of carboxy-DCFDA. The green fluorescence quickly appears in the capillaries and interstitial spaces but does not appear to enter the myocytes. The excitation power and gain of the microscope was reduced to prevent saturation in the 20 min image(D) while the gain and excitation power was constant for A-C. The blue autofluorescence, predominantly from NAD(P)H, is brighter in oxidative fibers(18). The red scale bar in figure A is 100 μ m in length.

Figure 2.

Time course of the dye fluorescence channel amplitude in the vascular space, extracellular space, and a cell during a carboxy-DCFDA infusion in mouse skeletal muscle, *in vivo*. The intercellular space and the cell cytosol was determined from the cellular NAD(P)H fluorescence.

Figure 3.

Mouse skeletal muscle 2-photon excitation fluorescence image 10 minutes after systemic injection of DCFA-SE. These data were collected with the Zeiss META spectral analysis system. Representative M_{DS} and M_{BS} spectra from mouse skeletal muscle collected are presented in the spectral panel. Spectra were collected using ROI located within the center of a cell, M_{BS} , or from a vascular compartment, M_{DS} . 4 averages were used to improve the signal to noise of the spectral data. The NAD(P)H and DCFA-SE images were calculated using I_{BF} and I_{DF} respectively for signal intensity. The combined fitted image is the linear combination of the NAD(P)H and DCFA-SE image. These data are consistent with the notion that most of the fluorescence was located in the extracellular space of the tissue. The residuals of this

analysis for a single image were less than 10%. The asterisks indicate motion artifacts from breathing that are most obvious in the combined image. The time required to collect these images contributed to the motion artifacts observed.

Figure 4.

Two photon excitation fluorescence image of the *in vivo* rabbit TA 25 minutes after arterial infusion of CFDA-SE. The individual images are presented as described in Figure 3. Again, fluorescence was limited to the extracellular space even with a direct arterial diffusion. The venous structure (v) was identified by the late enhancement of this vascular structure.

Figure 5.

Effective dyes for 2 photon excitation fluorescence studies in the mouse skeletal muscle *in vivo*. Syto-Green was used to highlight nuclei. Syto-Green was found to effectively label perivascular as well as nuclei deep in the muscle tissue and other organs. ANEPPS-8 was found to effectively label the vascular epithelium, *in vivo*. Acridine Orange provided a nuclear and more general tissue labeling probe that could prove useful as an inert reference for quantitative studies. The NAD(P)H images are not provided for ANEPPS-8 or Acridine Orange to conserve space, no significant interference of these probes with the NAD(P)H signal was observed. Dye concentrations were calculated to be on the order of 50 μM when fully mixed with the vascular

volume of the mouse. Observations in the liver and kidney revealed excellent labeling in these tissues with these probes. All injections were made via venous cannulation.

Table 1
Half Life of non-fluorescent form of probes in porcine plasma.

AM Probes	Probe type	$\frac{1}{2}$ life in plasma (min) ¹	$\frac{1}{2}$ life in buffer (min)	n
SNARF 1-S (invitrogen #S22801)	pH	< 1.0	>60	3
SNARF 1 (invitrogen #C-1271)	pH	< 1.0	>60	3
Carboxy-DCFDA (invitrogen #C-369)	pH	1.0	>60	3
Carboxy dichlorofluorescein Succinimidyl (custom synthesis)	Cell tracker	2.5	>60	3
CFDA-SE (invitrogen #C1157)	Cell tracker/pH	10	>60	3
Fluo 4 (invitrogen #F14202)	Ca++	7.5	>60	3
Fluo 4jo (succinimidyl) (Sigma #77860)	Ca++	6	>60	3
Fluo 3 (invitrogen #F-1241)	Ca++	7.3	>60	3
Indo 1 (invitrogen #I-1203)	Ca++	2.0	na	3
Ca Green (invitrogen #C-3011MP)	Ca++	5.5	>60	3
Mag Fura 2 (invitrogen #M-14206)	Mg++	< 1.0	>60	1

¹ Half life linearly corrected for plasma dilution.