Development of an Immunologically Tolerated Combination of Fluorescent Proteins for In vivo Two-photon Imaging

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Combinations of fluorescent proteins (FPs) are routinely used for multi-parameter in vivo imaging experiments to visualize tagged proteins or cell populations of interest. Studies involving FPs are often limited by spectral overlap, toxicity, relative quantum efficiency, and the potential for immunological rejection upon transfer into a non-tolerant recipient. Here we evaluate the immunologic visibility of several commonly used FPs by the murine immune system and identify a spectrally compatible, immunologically tolerated combination of FPs well suited for in vivo two-photon imaging.

Two-photon laser scanning microscopy (TPLSM) has vertically enhanced our understanding of biological systems by allowing real-time deep tissue imaging. Two-photon microscopes are usually equipped with titanium-doped sapphire (Ti:sapphire) lasers that generate pulsed near-infrared light. Using a Ti:sapphire laser, excitation of a fluorophore is achieved when two photons are absorbed simultaneously. This ensures that the probability of excitation is highest in the focal plane, thus minimizing undesirable excitation above and below this plane. Moreover, use of near-infrared light allows deeper penetration into tissues than can be achieved with conventional single photon lasers. In fact, imaging depths of greater than 1 mm have been reported in the literature. Given these advantages and others, TPLSM has become an invaluable microscopy technique for imaging living systems, and its use has expanded greatly in recent years.

Because TPLSM allows design of 4-dimensional (4D) multi-parameter in vivo imaging experiments, it is routine to utilize panels of fluorescent probes, proteins, etc. to visualize multiple cell populations and/or structures of interest. This requires optimization to ensure compatibility among the selected fluorophores. Use of fluorescent proteins became a mainstay in the imaging community following the discovery of green fluorescent protein (GFP), which was isolated from the jellyfish Aequorea Victoria. The palette of available fluorescent proteins (FPs) has expanded considerably since then to include derivatives of GFP such as cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). FPs have also been isolated from other species such as DsRed. There are now more than 50 different FPs available with spectral properties ranging from blue to far red. However, it is important to consider that not all FP combinations are ideal, and some FPs can be toxic or even immunologically rejected upon transfer into a non-tolerant host. We therefore set out in this study to evaluate the immunologic visibility of several commonly used FPs and to identify a combination of FPs that are bright, non-toxic, spectrally separable, and immunologically tolerated following adoptive transfer.

At present, it is standard practice in two-photon imaging experiments to adoptively transfer FP-tagged cells from a FP-positive donor into a genetically identical FP-negative recipient. Because FPs are non-self, adoptive transfers have the potential to result in a FP-specific immune response. To evaluate the immunologic “rejectability” of three commonly used FPs (i.e., CFP, GFP, and DsRed), we set up an adoptive transfer paradigm to monitor FP rejection over time. This was accomplished by crossing mice that express CFP, GFP, or DsRed with CD8+ T cell receptor (TCR) transgenic mice that recognize the glycoprotein (GP), amino acids 33–41, of lymphocytic choriomeningitis virus (LCMV) presented in H-2Dd (referred to as P14 mice). The resultant F1 crosses yielded P14 T cells expressing CFP, GFP, or DsRed under the β-actin promoter.

These cells were isolated from the spleen, and 5,000 of each population were adoptively transferred into C57BL/6J (B6) recipients. One day later the mice were challenged intraperitoneally with lymphocytic choriomeningitis virus (LCMV) Armstrong strain, which results in an acute viral infection that is cleared in 8–10 days. Following...
Having generated two new transferrable FP reporter lines, we mostly evaluated the ease with which the FPs expressed in these lines could be detected and spectrally separated following two-photon illumination in a living tissue. This was accomplished by conducting in vivo multi-parameter TPLSM experiments with the two-photon laser tuned to 920 nm. CAG-mOrange and CAG-mTFP1 mice were crossed with P14 mice to generate populations of traceable LCMV-specific CD8+ T cells. Naïve CD11c-YFP mice that express YFP in dendritic cells were seeded intravenously with naïve mTFP1 and mOrange P14 cells (5,000 of each population) and infected one day later with LCMV. For comparison, we seeded another group of CD11c-YFP mice with CFP and GFP P14 cells. CFP, GFP, and YFP are used for multi-parameter two-photon imaging experiments, but the color combination is spectrally sub-optimal. Separation of the emission spectra using dichroic mirrors at 458, 495, and 525 nm revealed a considerable degree of overlap between the FPs (Fig. 3A, upper panel). This overlap becomes even more apparent in an unprocessed four-color TPLSM data set captured in the intact lymph node of a LCMV-infected CD11c-YFP mouse seeded with CFP and GFP P14 cells (Fig. 3A, middle panel; Movie 1). Images corresponding to the four channels of the NDD4 detector array showed the original fluorescence emissions for second harmonic (channel 1), CFP (channel 2), GFP (channel 3), and YFP (channel 4). Note the considerable degree of fluorescence overlap between the channels, particularly in the merged image. Application of a spectral unmixing algorithm to the data set resulted in better separation between the channels, but some overlap remained (Fig. 3A, lower panel; Movie 1). This is evidenced by the failure to generate four pure channels (white, blue, green, red) in the resultant merged image (Fig. 3A, lower panel).

By comparison, the emission spectra for mTFP1, YFP, and mOrange showed a much better degree of separation than the CFP/GFP/YFP color combination (Fig. 3B, upper panel), and using optimally placed dichroic mirrors at 458, 510, and 561 nm, the resultant original TPLSM data set revealed a lower degree of overlap between the channels. For example, pure blue and red coloration was observed in the original merged image (Fig. 3B, middle panel; Movie 2). Spectral unmixing further improved the quality of this data set and generated perfect separation of the data into four distinct channels (white, blue, green, red) (Fig. 3C, lower panel; Movie 2). These data demonstrate that mTFP1, YFP, and mOrange are a well-suited color combination for multi-parameter two-photon imaging experiments.

In conclusion, FPs have become an essential component of many in vivo two-photon imaging experiments, and FP-tagged cells are often transferred into recipients despite the potential for immunological rejection of the foreign FP. In this study, we set up an adoptive transfer paradigm to evaluate the rejectability of several commonly used FPs. Surprisingly, we observed that only 1 of the 6 FPs tested (DsRed) was rejected upon adoptive transfer into a B6 recipient, consistent with a previous study showing that a cytotoxic lymphocyte response can be generated against this protein. We further demonstrated that a humoral response is also mounted against DsRed and immunological tolerance. Interestingly, the dominant DsRedT3 epitope (SSLQDGCFI) previously reported to be recognized by CD8+ T cell receptor recognition. Alternatively, we postulate that rejection of DsRed in non-tolerant mice is linked to its tetrameric structure and/or ability to aggregate, as monomeric deri-
Figure 1 | Evaluation of the immune reaction to commonly used fluorescent proteins. (A, B) Naive B6 mice (n = 5 per group) were seeded i.v. with 5,000 fluorescent protein (FP)-expressing P14 CD8+ T cells and infected one day later i.p. with 2 × 10^5 PFU LCMV Arm. The percentage of FP-expressing CD8+ T cells were monitored in the blood at the indicated time points. Representative flow cytometric plots gated on CD45+ CD8+ T cells are shown in panel A. Pink boxes denote the percentage of FP-expressing CD8+ T cells. Note that DsRed+ P14 cells are completely eliminated by day 28. In panel B, data are plotted over time as the percentage of FP+ P14 cells (mean ± SD) remaining relative to day 7. The colored lines represent fitted curves depicting P14 decay over time. (C) The upper panel shows a schematic of the NSE-DsRedE2 construct used to generate B6 NSE-DsRedE2 transgenic mice. The lower bar graph shows that absolute quantity of DsRed mRNA in 1 µg of total RNA harvested from the denoted tissues. Data are represented as mean ± SD (n = 4 mice). (D) 5,000 DsRed+ P14 cells were adoptively transferred into naive B6 or NSE-DsRed mice (n = 5 mice per group) and infected one day later with LCMV Arm. The fate of GFP+ P14 cells in B6 mice served as a control for this experiment. Normalized percentages (mean ± SD) are plotted over time in a manner identical to that described in panel B. DsRed+ P14 cells are maintained in NSE-DsRed but not B6 mice. (E) ELISAs were performed to detect anti-DsRed antibodies. Representative ELISA data are plotted for serially diluted sera obtained at day 15 post-infection from B6 and NSE-DsRed mice seeded with DsRed+ P14 cells (n = 5 mice per group). Anti-DsRed and anti-GFP antibodies served as positive and negative controls, respectively. (F) Curves analogous to those shown in panel E were generated at the denoted time points. Fluorescence emission from the third dilution is plotted versus time. Data are represented as mean ± SD (n = 5 mice per group). B6 mice seeded with DsRed+ P14 cells generate anti-DsRed antibodies, whereas NSE-DsRed mice do not. All data are representative of two independent experiments.
Tetrameric or aggregate DsRed may promote humoral immunity by eliciting multi-valent stimulation of B cell receptors following release from dead or dying cells. DsRed aggregation may also facilitate uptake by antigen presenting cells and presentation to T cells. To obtain a spectrally compatible color palette for multi-parameter two-photon imaging experiments, we generated two new transgenic FP reporter mice: CAG-mTFP1 and CAG-mOrange. mTFP1 is very photostable and has one of the highest quantum yields of any FP. mOrange is also photostable and has the added advantage of being photoconvertible to a far-red protein. Both proteins were immunologically tolerated by the B6 immune system and performed optimally in a TPLSM experiment when imaged with YFP. This contrasted with the CFP/GFP/YFP combination which retained some spectral overlap even after unmixing. Another advantage of using mTFP1/YFP/mOrange is that all three FPs (plus collagen) were excited with a single wavelength of two-photon light (920 nm), which has advantages over other FP combinations that require dual lasers set to different wavelengths.

Collectively, our studies should help advance future TPLSM experimentation by providing a compatible, immunologically-tolerated FP combination for adoptive transfer studies.

**Methods**

**Mice.** C57BL/6J (B6/J), B6.129(ICR)-Tg(CAG-ECFP)Ck6Nagy/J (actin-CFP), C57BL/6-Tg(CAG-EGFP)1Osbr/J (actin-GFP), B6.Cg-Tg(CAG-DsRed*2MST)1Nagy/J (actin-DsRed), B6.Cg-Tg(CAG-mRFP1)1F1Hadj/J (actin-mRFP1), B6 CD11c-YFP mice, B6 Thy1.1 DbGP33–41 TCR-tg (Thy1.1 P14), and B6 DbGP33–41 TCR-tg (P14) mice were bred and maintained under specific pathogen-free conditions at the National Institute of Health (NIH). B6, actin-CFP, actin-GFP, actin-DsRedT3, and actin-mRFP1 were originally purchased from The Jackson Laboratory. CD11c-YFP mice were generously provided by M. Nussenzweig (Rockefeller University, New York, NY), were backcrossed for three additional generations to obtain mice on a pure B6/J background. Fluorescent protein-expressing P14 cells were generated by setting up F1 crosses between P14 (or Thy1.1 P14) mice and each of the aforementioned actin transgenic reporter mice.

**Transgenic mouse generation.** All transgenic mice were generated by the National Institute of Mental Health (NIMH) Transgenic Core Facility. To generate mice expressing DsRed Express2 under the neuron specific enolase promoter (NSE-DsRedE2) a cDNA construct was made by replacing the cytomegalovirus (CMV) promoter.
promoter in the pCMV DsRed-Express2 vector (Clontech) with the murine NSE promoter. The NSE promoter sequence (2060 bp) was PCR amplified using primers (fwd: 5'ATATATATTAATGTTGTTAAACCTTCGATTCCG3'; rev: 5'TATATGCTAGCTCGAGGACTGCAGACTCAG3') containing the AseI and NheI restriction enzyme sites. This promoter was cloned into the pCMV-DsRedE2 vector following removal of the CMV promoter. A rabbit beta-globin polyadenylation signal sequence (462 bp) was PCR amplified from the pCAG-GFP vector (Addgene) and inserted downstream of DsRed-Express2 cassette at NotI restriction enzyme site. Transgenic mice expressing monomeric teal fluorescent protein (mTFP1) under the chicken \(\beta\)-actin promoter were generated by first PCR amplifying the entire 708 bp mTFP1 coding region using primers (fwd: 5'ATATATGAATTCGCCACCATGGTGAGCGGCGG3'; rev: 5'ATATATCTCGAGTACTTGTACAGCTGTCGAGG3') containing the EcoRI and XhoI restriction sites. This cDNA was cloned into the same sites following removal of the GFP sequence from the pCAG-GFP vector (Addgene). The resultant plasmid was digested with SalI/HindIII, and a 2900 bp fragment containing the CMV early enhancer/chicken \(\beta\)-actin (CAG) promoter, mTFP1, and PolyA sequence was prepared for microinjection into the pronuclei of fertilized mouse eggs. To generate all transgenic mice, linearized constructs were injected into C57BL/6N eggs. Following selection of transgene positive founder lines, all mice were backcrossed onto the C57BL/6J background for

Figure 3 | Optimization of spectral reassignment and cellular tracking for in vivo two-photon imaging. (A, B) Graphs show the fluorescent protein emission spectra for the denoted FPs as well as second harmonic signal corresponding to collagen. The gray bars depict the dichroic mirrors used in the NDD4 external detector to separate the light into four distinct channels. The wavelengths of the dichroic mirrors are denoted above the gray bars. Naive CD11c-YFP mice were seeded with 5,000 CFP\(^+\) P14 cells/GFP\(^+\) P14 cells (panel A) or 5,000 mTFP1\(^+\) P14 cells/mOrange\(^+\) P14 cells (panel B) and infected one day later with LCMV Arm i.p. 3D time lapses were acquired in the lymph node at day 7 post-infection. The upper panels show representative unmanipulated maximal projections generated at a single time point that depict the lymph node capsule defined by second harmonic signal (white, channel 1) as well as a subcapsular region containing T cells and antigen presenting cells. Images depict the light collected by the four detectors (channels 1 through 4) in the NDD4 external detector box. The “merge” image represents the combination of the four channels (Ch). The lower panels show the same maximal projections following spectral unmixing using Leica Application Suite AF software. The boxes beneath the images provide the wavelengths of light captured by each channel of the NDD4 detector array. See corresponding Movies 1 and 2. Images shown in this figure are representative of two independent experiments.
Dipping objective (1.0 NA), a quad NDD4 external detector array, and a Mai Tai HP with a mixture of 95% O2 and 5% CO2. 3D time lapses were captured using a Leica SP5 then placed into a flow chamber perfused with 37°C PBS. Titering were established by performing plaque assays on Vero cells. Mice were infected with the Leica Application Suite AF software before final processing was performed in Imaris 7.3 Software (Bitplane).

**Graphs.** All graphing and curve fitting was performed using GraphPad Prism 6.0 (GraphPad Software, Inc.). The fitted curves shown in Figures 1B, 1D, and 2D were generated using the equation for a one phase decay: $Y = (Y_0 - Plateau)\times e^{-K\times X} + Plateau$. The curves in Figure 1E were generated using the equation for a sigmoidal dose-responses: $y = Bottom + (Top - Bottom)/(1 + 10^{(Exp\text{-}IC50-A\times X)})$.

**Animal ethics.** All experiments involving mice were approved by the National Institutes of Health Animal Care and Use Committee and conducted in accordance with the guidelines set forth by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

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Acknowledgments
This work was supported by National Institutes of Health (NIH) intramural program. D. Nayak was supported by a NIH Intramural Competitive Fellowship. We would like to thank Dr. James Pickel and the NIMH Transgenic Core Facility for their assistance in generating the new transgenic fluorescent protein reporter mice described in this manuscript.

Author contributions
S.G. and D.B.M. designed and completed the experiments. D.N. generated the transgenic mice. D.N. and D.B.M. wrote the manuscript. B.H.Z. suggested using OFP and mTFP1 for two-photon imaging, helped with data analysis, and prepared the figures/movies. All authors reviewed the manuscript.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Gossa, S., Nayak, D., Zinselmeyer, B.H. & McGavern, D.B. Development of an Immunologically Tolerated Combination of Fluorescent Proteins for In vivo Two-photon Imaging. Sci. Rep. 4, 6664; DOI:10.1038/srep06664 (2014).

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