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A Global Double-Fluorescent Cre Reporter Mouse

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Summary: The Cre/loxP system has been used extensively for conditional mutagenesis in mice. Reporters of Cre activity are important for defining the spatial and temporal extent of Cre-mediated recombination. Here we describe mT/mG, a double-fluorescent Cre reporter mouse that expresses membrane-targeted tandem dimer Tomato (mT) prior to Cre-mediated excision and membrane-targeted green fluorescent protein (mG) after excision. We show that reporter expression is nearly ubiquitous, allowing visualization of fluorescent markers in live and fixed samples of all tissues examined. We further demonstrate that mG labeling is Cre-dependent, complementary to mT at single cell resolution, and distinguishable by fluorescence-activated cell sorting. Both membrane-targeted markers outline cell morphology, highlight membrane structures, and permit visualization of fine cellular processes. In addition to serving as a global Cre reporter, the mT/mG mouse may also be used as a tool for lineage tracing, transplantation studies, and analysis of cell morphology in vivo. genesis 45:593-605, 2007. © 2007 Wiley-Liss, Inc.

Key words: mosaic; mT/mG; GPP; tdTomato; marker; membrane-targeted; recombination; cell morphology; lineage tracing

INTRODUCTION

Homologous recombination in mouse embryonic stem (ES) cells has greatly enhanced our understanding of gene function in mammalian tissues. Yet, conventional mouse knockouts may lead to embryonic lethality thereby precluding the study of gene function later in life. Moreover, pleiotropic effects complicate analysis in specific tissues. Conditional knockout methods using the Cre/loxP recombination system have allowed spatial and temporal control of gene knockout in mice (Nagy, 2000). In this system, Cre recombinase regulated by a tissue-specific and/or temporally-regulated promoter can excise essential loxP-flanked ("floxed") genes via intrachromosomal recombination to generate conditional knockouts (Gu et al., 1994). Over a hundred mouse Cre lines have been developed, each requiring characterization of the spatial and temporal pattern of Cre expression

(Nagy and Mar, 2001). This is generally accomplished using a Cre reporter transgene in which a marker gene (e.g. LacZ, GFP, CFP, or YFP) is expressed only following Cre-mediated recombination (Mao *et al.*, 2001; Soriano, 1999; Srinivas *et al.*, 2001). To assess the range of tissues in which Cre reporters are useful, it is important to label not only cells after Cre-mediated recombination but also nonrecombined cells. To this end, double reporter transgenes that express one marker (e.g. LacZ) in nonrecombined cells and another marker (e.g. alkaline phosphatase or GFP) in recombined cells were generated (Lobe *et al.*, 1999; Novak *et al.*, 2000).

The widely used double Cre-reporter lines, Z/AP and Z/EG (Lobe et al., 1999; Novak et al., 2000), make use of one enzyme-activated marker and one fluorescent marker for identifying cells in mosaic animals. In contrast, a double-fluorescent marker system would allow for visualization of recombined and nonrecombined cells without the addition of an exogenous enzymatic substrate, simplifying mosaic analysis especially in live tissues. While green fluorescent protein (GFP) (Chalfie et al., 1994) is widespread as a fluorescent marker in mice, a complementary red fluorescent protein (RFP) for in vivo mammalian studies has been difficult to obtain. DsRed, derived from coral Discosoma sp., was one of the first cloned RFPs and an excellent tool for in vitro studies (Baird et al., 2000; Matz et al., 1999). However, transgenic mice with ubiquitous DsRed expression have not been successfully generated, likely due to the toxicity of the molecule (see Discussion for recent development of transgenic mice with DsRed variants). It has been speculated that the oligomerization of DsRed to a tetramer and its long maturation time may be the cause for toxicity (Baird et al., 2000; Shaner et al., 2005). Recently, several new DsRed variants with a wide yellow-orange-red spectrum have been developed (Shaner

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et al., 2004). One of these RFP variants, tandem dimer Tomato (tdTomato), demonstrates photostability and brightness on par with or greater than that of enhanced GFP (EGFP) under ideal conditions. Moreover, tdTomato exhibits a short maturation time and folds equivalently to a monomer, which may minimize toxicity (Shaner *et al.*, 2004).

Here we describe a double fluorescent Cre recombinase reporter mouse, *mT/mG. mT/mG* expresses membrane-targeted tdTomato ("mT") prior to Cre excision and membrane-targeted EGFP ("mG") following Cre excision, thereby allowing live visualization and distinction of recombined and non-recombined cells. The combination of a strong and ubiquitous pCA promoter (Niwa *et al.*, 1991) and the *ROSA26* targeting locus (Soriano, 1999) enables bright fluorescent labeling of all tissues examined: red before and green after recombination. In addition, the localization of fluorescent proteins to membrane structures ("m") outlines cell morphology and allows resolution of fine cellular processes.

RESULTS

Generation of *mT/mG* Mice

The mT/mG expression construct is summarized in Figure 1. To develop the construct, we replaced the intervening gene sequence in a loxP-gene-loxP cloning vector (Sauer, 1993) with an N-terminal membranetagged version of tdTomato (mT) (Shaner et al., 2004). We next inserted an N-terminal membrane-tagged version of EGFP (mG) (De Paola et al., 2003) distal to the second loxP site (see Methods for construction of mTand mG). The resulting mT/mG cassette (loxP-mT-pAloxP-mG-pA) was cloned into an expression vector containing a CMV β -actin enhancer-promoter (pCA) for strong and ubiquitous expression of the double reporter cassette (Zong et al., 2005). The mT/mG expression construct was subsequently tested in vitro by transfecting COS cells with mT/mG with or without a Cre expression vector. In the absence of Cre, all successfully transfected cells were mT-positive, while cells transfected with both *mT/mG* and Cre were mG-positive. Both mT and mG exhibited equivalent membrane localization under fluorescence microscopy (data not shown).

Having validated the mT/mG expression vector in vitro, we inserted an FRT-flanked neomycin resistance gene (as a selectable marker) distal to the expression cassette and knocked the resultant construct into the *ROSA26* locus on chromosome 6 in mouse ES cells. *ROSA26* was chosen because it is a ubiquitously-expressed endogenous locus, thereby eliminating potential silencing effects of local chromatin structure (Soriano, 1999). It supports ubiquitous high-level expression of a single copy EGFP transgene under the control of a pCA promoter (Zong *et al.*, 2005). Moreover, homozygous *ROSA26* knock-in mice are viable without any phenotypic effects (Soriano, 1999). *mT/mG*-positive ES cells were identified by PCR (see Methods) and microinjected



FIG. 1. Schematic diagram of the *mT/mG* construct before and after Cre-mediated recombination. *mT/mG* consists of a chicken β -actin core promoter with a CMV enhancer (pCA) driving a loxP-flanked coding sequence of membrane-targeted tandem dimer Tomato (mT) resulting in tdTomato expression with membrane localization. After Cre-mediated intra-chromosomal recombination, the mT sequence is excised allowing the pCA promoter to drive expression of membrane-targeted enhanced green fluorescent protein (mG). Arrows denote the direction of transcription. Triangles represent loxP target sites for Cre-mediated recombination. PA denotes polyadenylation sequences. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

into blastocysts for generation of chimeras. Chimeric mice were crossed with wild-type mice for germline transmission.

Global Expression of tdTomato in *mT/mG* Mice

Heterozygous and homozygous mT/mG mice are fully viable and fertile without observable adverse phenotypes, demonstrating minimal mT toxicity in vivo with the afforded expression level. We assessed mT labeling in multiple tissues of mT/mG mice by live whole mount and paraformaldehyde-fixed cryosection analysis (Fig. 2a). We found strong red fluorescence in all tissues. While the degree of mT labeling varied between different cell types likely due to their inherent differences in protein synthesis, membrane trafficking, and metabolic properties, nearly all cells were labeled with the fluorescent marker. Moreover, mT outlines the plasma membrane of cells in all tissues observed, indicating that the pCA promoter and N-terminal membrane tag function appropriately. Live visualization of single mT-labeled cells and their fine processes was verified in cultures of primary neurons derived from *mT/mG* embryonic cortical caps (Fig. 2b). Finally, we determined that pCA-driven expression of marker genes is maintained despite increasing mouse age, as evidenced by a similar level of mT brightness when comparing older and younger tissues (Fig. 2c).

Cre-Dependent Expression of mG

To determine whether mT/mG functions effectively as a Cre reporter, we crossed mT/mG mice with *hprt*-



FIG. 2. Ubiquitous mT labeling in mT/mG mice prior to recombination. (a) Live whole mount and fixed tissue sections of various organs from an adult mT/mG mouse demonstrating ubiquitous mT labeling. (b) Examples of individual live mT-labeled cultured neurons derived from embryonic cortical caps of mT/mG mice. (c) mT/mG cerebella and olfactory bulb glomeruli reveal no difference in mT fluorescence between mice at 12 versus 42 weeks of age. Scale bars: (a) 200 µm, (b) 50 µm, (c) 200 µm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Cre mice (Tang *et al.*, 2002). Double transgenic progeny demonstrate complete replacement of mT with mG protein in all tissues examined (Fig. 3a), as expected by the ubiquitous expression of *bprt* (Tang *et al.*, 2002). While a small degree of overlap exists between the excitation and emission spectrums of EGFP and tdTomato (Shaner *et al.*, 2004), we observed minimal background emission from mT fluorescence in the green channel (Fig. 3b), demonstrating that mT and mG can function as complementary fluorescent reporters for identifying recombined and nonrecombined tissues.

Previous work from our laboratory has shown that single-copy cytoplasmic EGFP is sufficient for labeling single cells and neuronal processes (Zong *et al.*, 2005). Like mT, mG was readily visualized in live primary neurons and their processes in culture (Fig. 3c). To verify single cell resolution of single-copy mG in vivo, we generated small clones of mG-positive cells using an inducible Cre transgenic line, *actin-CreER* (Guo *et al.*, 2002). In this line, CreER protein is sequestered in the cytoplasm and is unable to catalyze DNA excision. Binding of tamoxifen (TM) to CreER permits nuclear translocation and recombination (Guo *et al.*, 2002). We injected pregnant mothers carrying mT/mG; actin-CreER embryos with 0.5 mg TM at embryonic day 13.5 (E13.5) and the pups were dissected at postnatal day 21 (P21), a time-point where development and elaboration of neuronal processes is largely complete in the mouse brain. The low dose of TM administered resulted in labeling of single neurons and glia. Analysis of fixed brain sections from these mice





FIG. 3

shows that fine axonal processes of olfactory sensory neurons projecting into the olfactory bulb are readily observed (Fig. 3d), signifying that mG is transported efficiently along axons. Moreover, fine projections of Bergmann glia in the cerebellum are strongly labeled (Fig. 3e). Immunostaining with an anti-GFP antibody further enhances these processes in mG-positive cells (data not shown). In summary, the above data show that mG is readily visualized in single cells and axonal projections in vivo.

To demonstrate tissue-specific expression of mG, we mated mT/mG mice with nestin-Cre transgenic mice (Petersen et al., 2002). As nestin is expressed in neural precursors (Lendahl et al., 1990), we observed mG-labeled cells in the spinal cord (Fig. 4a), choroid plexus (Fig. 4b), brain (Fig. 4c), and retina (Fig. 4d) but not in non-nestin-expressing cells like liver hepatocytes (data not shown). We also generated mosaic mice with the actin-CreER line. As anticipated, injection of 2 mg of TM at P7 followed by dissection at P21 reveals a mosaic and mutually exclusive pattern of mT and mG labeling in multiple tissues including the liver (Fig. 4e,e"), brain (Fig. 4f), and retina (Fig. 4g). The complementary pattern of mT and mG is particularly striking in liver hepatocytes (Figs. 4e-e") but less apparent in the brain (Fig. 4c,f) likely due to substantial intermingling of neuronal processes. Increasing the dose of administered TM results in a correspondingly higher proportion of mG labeling (data not shown).

Complementarity of marker expression was generated presumably because the 2-week period between Cre induction and analysis allows turnover of mT in cells that have undergone Cre-mediated excision. Examination of tissues at a shorter time interval reveals double-labeled cells (yellow cells; Fig. 4h,i), due to the rapid onset of mG labeling within 24 h following Cre induction (data not shown; Hayashi and McMahon, 2002) and the perdurance of mT mRNA and protein. We quantitatively assessed this lag in loss of mT fluorescence by dissecting *mT/mG;actin-CreER* mice 3 days or 7 days following Cre induction by TM injection. We quantified the proportion of mG-labeled hepatocytes that also retained mT. Hepatocytes were chosen as an example tissue for quantification as they represent an easily identifiable and uniform cell type. We observed a steady decline in double-labeled cells with an expected ~9.2-day interval (extrapolation of linear regression) for loss of mT in nearly all mG-labeled cells (Fig. 4j). We expect that the rate of mT loss after excision will likely vary from tissue to tissue based on cell type-specific mRNA and protein turnover properties.

In summary, these data demonstrate that mG labeling is Cre-dependent, is mutually exclusive from mT labeling, and can be generated by ubiquitous, tissue-specific, and inducible Cre transgenic lines.

Complementary Fluorescent Membrane Labeling at Single Cell Resolution

To test the versatility of mT/mG for analysis of cell morphology, we analyzed mT and mG labeling in mT/mG;actin-CreER mice dissected 2 weeks post-TM injection in multiple tissue types (epithelium, connective tissue, muscle, germline). A diverse array of epithelial cells can be identified by the fluorescent markers including cuboidal hepatocytes arranged in sheets and cords in the liver (Fig. 5a), squamous alveolar cells lining the airway septae of the lung (Fig. 5b), columnar enterocytes lining the intestinal villi (Fig. 5c,d), cuboidal and squamous epithelia of the hair follicles and epidermis (Fig. 5e), squamous endothelium lining the blood vessels of the kidney glomeruli (Fig. 5f), and cuboidal epithelium of the kidney tubules (Fig. 5g). Connective tissue including bone osteocytes/chondrocytes (Fig. 5h) and muscle cells of smooth (Fig. 5i), cardiac (Fig. 5j), and skeletal (Fig. 5k) varieties are readily visualized. Finally, gonadal tissues including cells in various stages of spermatogenesis and Leydig cells (Fig. 5l, asterisk) are clearly outlined by the membrane-targeted markers. Notably, all cells as indicated by the nuclear stain DAPI (in blue) are outlined by the fluorescence markers, indicating that at least in these tissues, our Cre reporter is ubiquitously expressed.

These images highlight a few key features afforded by a membrane-targeted double fluorescent reporter mouse line. First, the mT and mG markers are predominantly mutually exclusive in almost all tissues examined. The main exception is skeletal muscle (Fig. 5k), which appears yellow due to coexpression of mT and mG. This coexpression likely results from the multinucleate nature of muscle cells in which some nuclei have undergone Cre-mediated recombination while others have not. In contrast, mononucleate smooth muscle and cardiac muscle retain complementarity of mT and mG labeling (Fig. 5i,j). The infrequent yellow markings in other tissues likely represent membrane overlap (Fig. 5i, arrowheads) or perdurance of the mT protein. Second, mT and mG outline specialized membrane structures including the brush border of the intestinal epithelium

FIG. 3. mG labeling in mT/mG mice after Cre-mediated recombination. (a) Live whole mount and fixed tissue sections of various organs from an adult mT/mG; hprt-Cre mouse demonstrating global mG labeling. Images represent composite of red and green channels, indicating minimal red fluorescence. (b) Confocal images (1- μ m optical sections) of fixed tissue sections of mT/mG and mT/mG; hprt-Cre cerebella demonstrating complete transformation from mT to mG expression with the addition of hprt-Cre. The images on the top panels are taken with the same imaging conditions as those in the bottom panels, showing minimal background fluorescence of mT and mG into the opposite channels. (c) Example of individual live mG-labeled cultured neurons derived from embryonic cortical caps of mT/mG; nestin-Cre mice. (d) mG-labeled olfactory sensory neuron axons visualized in an olfactory bulb glomerulus (encircled) of a P21 mT/mG; actin-CreER mouse previously induced with tamoxifen. The arrow denotes an axon entering the glomerulus. (e) mG-labeled Bergmann glia cell bodies (arrows) and their processes (arrowheads) visualized in fixed cerebellar sections from the same mouse as (d). Scale bars: (a) 200 μ m, (b) 50 μ m, (c) 50 μ m, (d) 25 μ m, (e) 100 μ m.

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FIG. 4. Tissue-specific and inducible Cre-mediated expression of mG. (a) Live whole mount E17.5 mT/mG;nestin-Cre mouse showing mG labeling in neural tissue (spinal cord) and mT labeling in surrounding non-neural tissues. (b-d) Fixed tissue section of adult mT/mG;nestin-Cre brain and retina showing mG labeling in neural progenitor-derived tissue including the choroid plexus (b), cerebral cortex (c), and retina (d). (e-g) Fixed tissue sections of P21 mT/mG;actin-CreER (injected with 2 mg tamoxifen at P7) mouse showing mosaic and complementary pattern of mT and mG labeling in the liver (e-e''), cerebral cortex (f), and retina (g). (h,i) Fixed tissue sections of liver hepatocytes from adult mT/mG;actin-CreER mice dissected at 3 days (h) or 7 days (i) following injection of 6 mg of tamoxifen. Perdurance of mT is evident by the presence of double-labeled (yellow) cells. (j) Quantification of the proportion of recombined (mG-labeled) cells that retain mT label (i.e. double-labeled or yellow cells) at the time points shown in (h) and (i). The data point at Day 3 and Day 7 represent an average from counting cells in 9–12 different high-power fields (error bars are SEM). The data point at Day 0 is a theoretical value as no recombination has occurred and all cells are mT-labeled at this time. Linear regression (best line-to-fit) intersects the x-axis at ~9.2 days. Scale bars: (a-i) 50 μ m.

(Fig. 5c, inset) and the highly absorptive proximal convoluted tubules of the kidney (Fig. 5g, arrows and label 1). This labeling pattern allows distinction of specific kidney tubules, as distal tubules and collecting ducts (Fig. 5g, label 2) do not possess a brush border and instead have more evenly distributed membrane-associated markers. Finally, the double-fluorescent system allows independent tracing of at least two lineages from progenitors. For example, labeling of intestinal crypt progenitors likely leads to the predominant expression of either mT or mG within progeny migrating apically to the villi (Fig. 5d), as in normal intestinal epithelium development. In summary, these data reveal that mT/mG serves as a global Cre reporter with single cell resolution. Moreover, the double fluorescent membrane-tar-

geted system permits tracing of lineage and characterization of cell morphology in vivo.

Fluorescence-Activated Cell Sorting of mT- and mG-labeled cells

Our histological analysis of various tissues above revealed that nearly all cells expressed a marker gene, either mT or mG (Fig. 5). To test if these fluorescently labeled cells can be detected by fluorescence-activated cell sorting (FACS), we isolated leukocytes from the spleen, thymus, and peripheral blood of wild-type, mT/mG, and mT/mG; bprt-Cre mice. Both mT and mG were detectable using FACS. However, the fluorescence intensity of mG was greater than mT due to technical limita-



FIG. 5. Mosaic and complementary expression of mT and mG at single cell resolution. Tamoxifen injection of adult *mT/mG;actin-CreER* mice results in mosaic expression of mT and mG in multiple tissue types (epithelial, connective, muscle) deriving from all three primary germ layers (ectoderm, mesoderm, and endoderm). Representative 1-µm optical confocal section images are shown. See text for details. Shown are liver hepatocytes (a), lung alveoli (b), intestinal villi (c) and crypts (d), skin (e), a kidney glomerulus (f), kidney tubules (g), bone (h), smooth muscle of the bladder in transverse and cross-section (i), cardiac muscle (j), skeletal muscle (k), and testes (l). Inset of (c) shows the brush border of labeled intestinal epithelium at 1000X magnification. Arrows signify the brush border of proximal convoluted tubules (g). Arrow-heads in (j) denote yellow labeling at unresolvable intercalated discs (junctions between individual mT-labeled and mG-labeled cardiac cells). Asterix in (l) denotes Leydig cells of the testes. (1) proximal convoluted tubule, (2) distal convoluted tubule or collecting duct. Red: mT fluorescence; Green: mG fluorescence; Blue: DAPI staining. Scale bars: 25 µm.

tions, in particular, the lack of a proper excitation laser for tdTomato (peak excitation 554 nm). When analyzing thymocytes from an mT/mG mouse with a 488 nm excitation laser, >95% of the cells were identified as mT-positive (Fig. 6a,b). As predicted, mT/mG; bprt-Cre mice exhibit a near complete absence of mT-positive thymocytes with \sim 88% of cells labeled with mG instead (Fig. 6c). In this sample, around 10% of cells can be classified as mGnegative, but they likely represent a population with a lower level of mG labeling (Fig. 6c, black gate) that cannot be accurately separated from the unlabeled cells using FACS. In fact, visual inspection of *mT/mG;bprt-Cre* thymocytes stained with nuclear marker DAPI under a fluorescence microscope revealed that >95% of DAPIpositive cells expressed mG at varying levels (data not shown).

Using our FACS experimental conditions, we were not able to completely resolve the mT-positive (Fig. 6e) and -negative (Fig. 6d) populations of splenocytes. Accurate quantification of mT-positive splenocytes was difficult likely due to higher autofluorescence of splenocytes (Fig. 6d) compared to thymocytes (Fig. 6a). FACS analysis of *mT/mG;bprt-Cre* mice revealed that at least 82% of cells are mG-positive (Fig. 6f). Similarly, $\sim 87\%$ of leukocytes in the peripheral blood in mT/mG;hprt-Cre mice were measured by FACS to be mG-positive (data not shown). However, when splenocytes were examined under a fluorescence microscope, >95% of DAPI-positive cells were mG-positive (data not shown). As in the case of thymocytes, the disparity between the FACS measurements and visual quantification may represent inability of FACS to distinguish cells that express low levels of mG from unlabeled cells or to exclude dead cells. Finally, FACS of splenocytes from adult mT/mG;actin-CreER mice injected with 6 mg of tamoxifen greater than 2 weeks prior to analysis resulted in \sim 29% of sple-



FIG. 6. Fluorescence-activated cell sorting of mT and mG-labeled leukocytes. FACS analysis of leukocytes derived from the thymus (**a**–**c**) and spleen (**d**–**f**) of wild-type (a,d), mT/mG (b,e), and mT/mG;hprt-Cre (c,f) mice. Fluorescence signals were detected in the FITC and PE channels. For both thymus and spleen, the gates containing unlabeled cells (black) were defined to include close to 100% of cells in wt. samples. In mT/mG thymus, more than 95% of cells are excluded from the black gate and shift towards higher signal in the PE channel, showing they are mT-positive. In contrast, wild-type and mT-positive cells cannot be clearly distinguished in the spleen sample. The green gates, which include mG-positive cells, were defined to be mutually exclusive with black (unlabeled cells) and red (mT-positive cells) gates. Based on this gating, >80% of leukocytes are mG-positive in both lymphoid organs of mT/mG;hprt-Cre mice.

nocytes labeled with mG (data not shown). This lower proportion of mG-labeled cells is consistent with sporadic recombination catalyzed by inducible *actin-CreER*.

Brightness and Photostability of tdTomato In Vivo

Previous studies in our laboratory have suggested that Myc epitope-tagged DsRed2 cannot be visualized live in vivo as a single-copy transgene (Zong *et al.*, 2005). By contrast, mT permits live visualization in multiple mouse tissues (Fig. 2). As *mT/mG* represents the first reported tdTomato mouse line to our knowledge, we wanted to assess the brightness and photostability of tdTomato in comparison to EGFP, a fluorescent marker that has been used for in vivo studies for many years. Calculations of brightness based on extinction coefficient and quantum yield of free protein in solution of physiologic pH (7.4) demonstrate that tdTomato is almost 3-fold brighter than EGFP (Shaner *et al.*, 2004). In *mT/mG* mice, we observe qualitatively similar if not greater brightness of mT compared to mG under fluorescence microscopy with conventional filter settings.

In contrast to brightness, photostability measurements have demonstrated that EGFP is almost 2-fold more stable (Shaner *et al.*, 2004). However, these calculations were not based on focused laser illumination, which is a necessary for imaging tissues or cells using confocal microscopy. Qualitative observations of mT/mG tissue samples suggested that mT was less photostable than mG. To test photostability quantitatively, we focally illuminated cryosections of mT/mG and mT/mG; hprt-Cre olfactory bulb glomeruli containing olfactory receptor neuron axons for a total of 5 min at an optimal wavelength in the excitation spectrum of tdTomato or EGFP, respectively (see Methods). After each interval exposure period, we determined composite pixel intensity (Fig. 7a). To test photostability of fluorescent proteins within membranes closer to the cell body, we similarly illuminated hepatocytes. The relative brightness of mT and mG over time reveals that mT is less photostable than mG in both assays (Fig. 7b,c). However, mT still retains sufficient stability for basic imaging applications, as none of the images shown in this study required antibody staining for signal preservation. Furthermore, selective photobleaching of mT may be advantageous to obviate possible bleedthrough between the green and red channels when mG reporter expression is the primary point of interest. It is worth noting that although mG is more photostable than mT, the kinetics



FIG. 7. Photostability of mT and mG. (a) Representative images of an olfactory glomerulus from mT/mG (mT) and mT/mG;hprt-Cre (mG) mice after exposure to 568 nm or 488 nm light, respectively, under 400× confocal microscopy. Exposure time in seconds is listed above the images. (b) Relative brightness ± SEM of samples in (a) over time. Each plotting represents an average of three different olfactory glomeruli. (c) Relative brightness ± SEM of hepatocyte membranes of mT/mG;hprt-Cre (mG) and mT/mG (mT) mice after laser treatment over time as in (a). Each plotting represents an average of three different hepatocytes. Scale bars: 25 µm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of photobleaching differs between the two tested tissues above and should be assessed separately for any other tissue of interest.

DISCUSSION

Reporter mouse lines of Cre activity are essential tools for using the Cre/loxP system in mice. We have generated mT/mG, a double-fluorescent Cre reporter that expresses tdTomato prior to excision and EGFP following excision. These markers are driven by a ubiquitously active promoter and tagged with membrane localization sequences. Cre reporter activity was verified with ubiquitous, tissue-specific, and inducible Cre transgenic mouse lines showing membrane labeling at single cell resolution. In the following, we discuss new features of mT/mG in comparison with existing Cre reporters.

A significant advantage of the mT/mG mouse line is that it permits global expression of the reporter gene from a defined locus. Most Cre reporter lines are transgenic (e.g. Novak *et al.*, 2000) or utilize a weak endogenous promoter (Mao *et al.*, 2001) without guarantee of high expression in all cells. In contrast, we have utilized a strong, ubiquitous promoter (pCA) to drive mT/mG coupled with knock-in at the well-characterized *ROSA26* locus. We have shown that this scheme indeed allows expression of mT/mG's fluorescent markers in all tissues examined, and allows marker expression in nearly 100% of cells in high-resolution analysis of tissue sections. By placing our construct into a well-defined locus, we avoided effects of locus variation on reporter gene expression and accessibility to Cre-mediated excision (Novak *et al.*, 2000). The locus-dependent sensitivity to Cre excision may complicate analysis of Cre expression (Hebert and McConnell, 2000); a defined locus for Cre reporter gene integration that allows global expression alleviates potential variability.

The double-fluorescent nature of mT/mG also provides several useful features. Genotyping of mice becomes trivial as tail or whole body epifluorescence is sufficient to identify mT/mG mice. More importantly, the double-fluorescent system allows direct live visualization of both recombined and nonrecombined cells at single cell resolution, offering an internal control for phenotypic analysis of Cre-induced mosaic mutants and providing a second marker for lineage tracing applica-

tions. A potential drawback of the double-fluorescent system is possible bleedthrough between the fluorescent channels. We have assessed this in multiple tissues and observe minimal bleedthrough between the red and green channels using conventional filters in fluorescence and confocal microscopy. Moreover, the differing photostability of mT and mG permit selective photobleaching of mT, if bleedthrough becomes an issue for a particular application. A second caveat of the double-fluorescent system is the perdurance of the first marker in recombined cells, which may result in double marker-expressing cells. While this does not preclude reporter analysis simply by viewing the green channel, applications requiring distinction of mT- and mG-labeled cells need to take this into consideration. We have observed that dissection 2 weeks after recombination induction with actin-CreER is sufficient to observe minimal numbers of double-labeled cells in most tissues examined. In hepatocytes, we have observed that the time window is significantly shorter with an approximate 9-day interval being sufficient to avoid most transient double-labeled cells. The lag in mT loss may vary based on tissue type and should be assessed by individual researchers for their tissue of interest.

The viability and fecundity of mT/mG mice indicates that tdTomato is a suitable RFP for mammalian tissues in vivo. The generation of a RFP mouse has been long sought after given the enhanced tissue penetration of longer wavelength light (Winnard et al., 2006). The apparent toxicity of tetrameric DsRed (Shaner et al., 2005), the preeminent red fluorescent protein in previous years, had precluded the development of a RFP mouse until recently. Mutant versions of DsRed including DsRed.T3 with a shorter maturation time (Vintersten et al., 2004), mRFP1, a monomeric version of DsRed (Ding et al., 2005; Long et al., 2005; Zhu et al., 2005), and more recently tdRFP, a tandem dimer version of mRFP1 (Luche et al., 2007), have permitted the development of RFP mice. tdTomato is a new DsRed variant with increased brightness and photostability (Shaner et al., 2004). For instance, it has six times the brightness of DsRed.T3, eight times the brightness of mRFP1, and two times the brightness of tdRFP. In addition, tdTomato is over 10 times more photostable than mRFP1 (Bevis and Glick, 2002; Shaner et al., 2004). Our study here suggests that the absolute brightness of mT in vivo is comparable with that of mG, although mT is less photostable. Given that mice that globally express mT are viable and that tdTomato has better optical properties than other available RFPs, tdTomato will likely become a new red fluorescent marker of choice in mouse genetics.

mT/mG is the first Cre reporter mouse to our knowledge that expresses a membrane-targeted fluorescent protein. We attached a membrane tag to our reporter proteins to aid in visualization of fine processes, in particular those of neurons. Our own experience with single-copy cytoplasmic EGFP demonstrated strong dendritic labeling, but weaker axonal labeling in live tissues (Zong *et al.*, 2005). A previous report has shown that the 41 amino acid N-terminal MARCKS membrane tag allows strong labeling of axonal processes in vivo (De Paola et al., 2003). This may be due to the higher surface-to-volume ratio in fine processes. Our results confirm that axonal processes are strongly labeled with the N-terminal MARCKS membrane tag resulting in strong labeling in olfactory glomeruli and the axon-rich molecular layer of the cerebellum. Single cell analysis confirms axonal labeling on par with if not better than our previous cytoplasmic version (Zong et al., 2005). On the other hand, dendrites appear less well labeled with mG than with cytosolic EGFP. This difference may be due to the lower surface-to-volume ratio of dendrites compared to axons. Moreover, cell counting of mG-labeled cells becomes more difficult as cell bodies are less prominently labeled compared to cells expressing cytosolic EGFP in complex tissues such as the brain. In many other tissues, however, the cellular outline provided by membrane-targeting affords a three-dimensional assessment of cell morphology that is not easily discerned from cytoplasmic marker localization. For example, membrane structures like the brush border of intestinal villi and the kidney tubules are nicely highlighted by mT or mG. Together, our results indicate that membrane localization of a fluorescent marker is highly effective and perhaps superior to cytoplasmic localization for axonal visualization and outlining cell morphology. While we used two different membrane tags for tdTomato and EGFP (see Methods), we observed no major difference in cellular localization in vitro or in vivo in almost all tissues examined, suggesting that the first 8 amino acids of the modified MARCKS N-terminus are sufficient to confer membrane localization.

In addition to its utility as a Cre reporter line, we anticipate a wide array of applications of mT/mG. In conjunction with tissue-specific and inducible Cre transgenic lines and conditional knockout genes, mT/mG can be used to trace lineage and perform single or multi-cell mosaic analysis due to the permanent genetic change that occurs with Cre-mediated excision. Moreover, the membrane-targeted markers may allow tracing of axons and other fine processes. Finally, the double-fluorescent system permits in vivo imaging of endogenous cells within mT/mG; *Cre* mice or transplanted mT- or mG-labeled cells derived from these mice. We anticipate that mT/mG will be a useful addition to the mouse geneticists' toolbox.

METHODS

Generation and Testing of the *mT/mG* Targeting Construct

Membrane-tagged EGFP (mG) was a gift from P. Caroni (Friedrich Miescher Institute, Basel, Switzerland) (De Paola *et al.*, 2003). It contains 41 amino acids from protein MARCKS with the third and fourth amino acids mutated to cysteines (MGCCFSKTAAKGEAAAERPGEAA-VASSPSKANGQENGHVKV). This sequence was fused to EGFP (Clontech) without its N-terminal methionine via a three amino acid-linker (GSV). The endogenous MARCKS is myristoylated on N-terminal glycine (Stumpo *et al.*, 1989), while the introduced cysteines, at positions 3 and 4 act as palmitoylation sites (Wiederkehr *et al.*, 1997). The plasmid encoding tdTomato was obtained from R. Tsien (Univ. of California, San Diego) (Shaner *et al.*, 2004). Membrane-tagged tdTomato (mT) was constructed by fusing the first 8 amino acids of the MARCKS sequence above (MGCCFSKT) to the N-terminal methionine). The fusion was constructed by PCR and it contains no additional nucleotides between the 8 amino acid membrane tag and the beginning of the tdTomato. The mT construct was tested in transiently transfected COS cells to ensure membrane localization of the marker proteins (data not shown).

The mT/mG construct was assembled within a modified pBluescript II expression vector (pBluescript II') with a SalI-SpeI-XhoI-SacII-NdeI multi-cloning site. The SpeI-XhoI restriction fragment of p302 (GenBank accession number U51223, a gift from Dr. B. Sauer) (Sauer, 1993) was subcloned into pBluescript II'. The Xhol-BamHI restriction fragment of p302 was subcloned into the standard pBluescript vector, and the BamHI site was modified to a SacII site with oligonucleotides. The XhoI-SacII restriction fragment was introduced into pBluescript II' to make Sall-loxP-pA-loxP-SacII-NdeI (p302-1). Restriction sites BamHI and XhoI were introduced into the 5' and 3' ends of mT by PCR and this PCR fragment was subcloned into pGEM-T (Promega), sequenced, and then introduced into p302-1 to make loxP-mT-pA-loxP. Restriction sites SacII and NdeI were introduced into the 5' and 3' ends of the mG-pA by PCR. This PCR fragment was subcloned into pGEM-T, sequenced, and then introduced into the loxP-mT-pA-loxP vector to make loxP-mTpA-loxP-mG-pA'. Finally, the 150-bp SacII fragment of the GFP N-terminus region was subcloned into loxP-mT-pAloxP-mG-pA' to make loxP-mT-pA-loxP-mG-pA (mT/mG) flanked by the restriction sites SalI and NdeI. The mT/mG sequence excised with Sall and NdeI was subcloned into an expression vector containing a CMV chicken βactin enhancer-promoter (pCA-HZ2) (Zong et al., 2005) by blunt end ligation. Orientation of pCA-mT/mG was verified by BamHI restriction digest.

The *pCA-mT/mG* construct was tested by transient transfection with or without a Cre expression plasmid in COS cells. Cells were observed for labeling 48–96 h post-transfection under an inverted fluorescence microscope (Zeiss). Following verification of construct function in vitro, an FRT-flanked Neo cassette (selectable marker) was subcloned distal to the *mG-pA* in *pCA-mT/mG* via EcoRI and AscI restriction sites added to the 5' and 3' ends of pL451 (NCI) (Liu *et al.*, 2003). The resulting vector was sequenced, cut with PacI and AscI, and subcloned into the *ROSA26* targeting vector (Srinivas *et al.*, 2001).

Generation of *mT/mG* Knock-In Mice

The *mT/mG* targeting construct was linearized with KpnI, and ES cell targeting was performed by the Stan-

ford Transgenic Facility using R1 mouse ES cells. Following G418 selection, positive clones were determined by two sets of PCR. The 5' PCR was performed as previously described (Zong et al., 2005) to generate a 1.5-kb positive fragment. The 3' PCR generated a 6-kb fragment with the following primers: mTG1 5'-CTTGGCGGCG-AATGGGCTGACCG-3' and Rosa9 5'-GGGGGAAAATTTT-TAATATAAC-3' (Zong et al., 2005). Two positive clones were expanded and injected into C57BL/6J blastocysts to generate chimeric mice. Germline transmission was verified by PCR as previously described (Zong et al., 2005). F1 mT/mG mice were either intercrossed to generate homozygous mice or crossed to hprt-Cre (Tang et al., 2002), nestin-Cre (Petersen et al., 2002), or actin-CreER (Guo et al., 2002) mice for Cre reporter analysis.

Tissue Preparation and Histology

All animal procedures were based on animal care guidelines approved by Stanford University's Administrative Panels on Laboratory Care (A-PLAC). For Cre induction with actin-CreER, pregnant mothers (for embryonic labeling) or postnatal mice were injected intraperitoneally with 0.5-8.0 mg of tamoxifen (Sigma) dissolved in corn oil. For whole-mount organ analysis, mice were sacrificed by cervical dislocation and organs were isolated and imaged with a CCD camera (Diagnostic Instruments) mounted on a fluorescence microscope (Zeiss). For cryosection preparation, tissues were isolated from anesthetized mice perfused with cold 4% paraformaldehyde (PFA; Sigma) in 0.1 M phosphate buffer saline (PBS; Sigma), fixed 6-24 h in 4% PFA at 4°C, cryoprotected in 30% sucrose overnight at 4°C, and embedded in OCT (Tissue-Tek). Ten-micrometer sections were obtained using a Leica cryostat. Slides were washed three times with PBS, treated with DAPI (Molecular Probes), washed again, mounted in Gel/Mount (Biomeda Corp.) and imaged as above or with confocal microscopy (Bio-Rad or Zeiss). Neither mT nor mG required immunostaining for visualization. To assess mG signal improvement with immunostaining, sections were treated with chicken anti-GFP antibody (1:500; Aves Labs) as previously described (Muzumdar et al., 2007). Anti-GFP antibodies do not cross-react with tdTomato, as these proteins are from different origin.

Cell Dissociation and Culture

For analysis of single mT- and mG-labeled cells, neuronal cultures derived from E17.5 cortical caps were prepared. Cells were isolated and dissociated as previously described (Zong *et al.*, 2005). Cortical neurons were cultured in neurobasal medium (Invitrogen) supplemented with N2 (Invitrogen), I-glutamine (Invitrogen), and penicillin-streptomycin (Invitrogen) and imaged under an inverted fluorescence microscope (Zeiss) after process elaboration 72–96 h later.

Fluorescence-Activated Cell Sorting

Spleen and thymus were isolated from wild-type, mT/mG and mT/mG; bprt-Cre mice, and blood cells were dissociated between two frosted microscope slides (VWR), passed through a 40-um nylon filter (Falcon) to remove doublets and cell clumps, washed with PBS, fixed for 3-5 min in cold 4% PFA, and stored in PBS at 4°C before sorting. Peripheral blood cells from tail or neck veins of anesthetized mice were collected in eppendorf tubes containing EDTA (Sigma). These samples were also filtered, washed in PBS, and fixed with 4% PFA prior to sorting. Cells were analyzed on a Flasher II Diva Digital (modified FACStar) sorter at the Stanford Shared FACS Facility. 100,000 events were collected for each sample. Due to technical limitations, a 488-nm excitation laser was used for both mT and mG excitation. The wavelength of 488 nm falls within the peak excitation of EGFP but only at \sim 25% of peak excitation for tdTomato. Data were analyzed using FlowJo FACS analysis software. Cells were gated for singlets (using forward scatter area vs. height), for leukocytes [based on size (forward scatter) and complexity (side scatter)], and fluorescence (FITC and PE) without compensation.

Analysis of mT and mG Photostability

To compare fluorescence protein photostability in vivo, olfactory bulb glomeruli on 10 μ m brain sections were focally illuminated under 40× magnification with excitation wavelengths of 568 nm and 488 nm for mT and mG, respectively, for five minutes. One-micrometer optical sections were imaged by confocal microscopy (Bio-Rad) at 0, 10, 30, 60, 120, 180, and 300 s after the onset of illumination. The same procedure was applied to liver sections. To calculate photostability, average pixel intensity of a glomerulus or a hepatocyte membrane was measured across images obtained at the above time points using the NIH ImageJ program. This was repeated for a total of three glomeruli and three hepatocyte membranes to generate a composite average at each time point. Graphs were generated using Microsoft Excel.

Note added in proof: mT/mG mice have been deposited to the Jackson Laboratory for distribution under Jackson Stock Number 007576.

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