ARTICLES

Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system

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Detailed analysis of neuronal network architecture requires the development of new methods. Here we present strategies to visualize synaptic circuits by genetically labelling neurons with multiple, distinct colours. In *Brainbow* transgenes, Cre/*lox* recombination is used to create a stochastic choice of expression between three or more fluorescent proteins (XFPs). Integration of tandem *Brainbow* copies in transgenic mice yielded combinatorial XFP expression, and thus many colours, thereby providing a way to distinguish adjacent neurons and visualize other cellular interactions. As a demonstration, we reconstructed hundreds of neighbouring axons and multiple synaptic contacts in one small volume of a cerebellar lobe exhibiting approximately 90 colours. The expression in some lines also allowed us to map glial territories and follow glial cells and neurons over time *in vivo*. The ability of the Brainbow system to label uniquely many individual cells within a population may facilitate the analysis of neuronal circuitry on a large scale.

Cajal revolutionized neurobiology when he used Golgi's silver stain to label small numbers of neurons in their entirety, thereby identifying the cellular elements of neural circuits. The small number of labelled cells, however, was also a limitation because quantitative information such as divergence and convergence at synaptic relays was inaccessible. Efforts are presently underway to produce connectivity maps in which multiple, or even all, neuronal connections are rendered[1–3]. Building such 'connectomic' maps would be more straightforward with the equivalent of a multicolour Golgi stain that would allow many neurons within a single sample to be individually identified by virtue of a large number of cell-specific labels. In the same way that a television monitor encodes colour space by mixing three primary channels—red, green and blue—the combination of three (or more) coloured dyes can generate many different hues[4]. Multiple spectral variants of fluorescent proteins now exist[5] and are ideal labels for this purpose.

Combining two XFPs in one animal and stably expressing them in a mosaic manner has been achieved by various strategies, including the generation of chimaeric mice mixing stem cells of two colours[6]; the selection and crossing of mouse lines showing variegated expression of yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP)[7,8]; or a genetic scheme reconstituting split XFP genes by crossing over[9]. The first two approaches are, however, cumbersome, and all three generate only a limited palette of colours.

To circumvent these limitations, we exploited the advantages of the widely used Cre/*lox* recombination system[10], which can switch on gene expression by DNA excision, inversion, or interchromosomal recombination[9–11]. We designed two genetic strategies, called Brainbow, for stochastic expression of multiple fluorescent proteins from a single transgene. *Brainbow-1* uses Cre-mediated excision between pairs of incompatible *lox* sites, alternated to create mutually exclusive recombination events. In *Brainbow-2*, Cre inverts DNA segments delimited by *loxP* sites in opposite orientation, positioned in tandem to generate several recombination outcomes. The differential expression of multiple copies of these constructs generates XFP mixtures, allowing the labelling of individual neurons and glia with as many as 90 distinguishable colours.

Strategies for mosaic expression of multiple genes

Stochastic recombination using incompatible *lox* sites. *Lox* variants have been developed with mutations that prevent them from recombining with the canonical *loxP* site, while allowing them to recombine specifically with identical *lox* sequences[10,12]. We reasoned that by alternating such variant *lox* sites with canonical *loxP* in the same construct, we could force Cre to choose between two mutually exclusive excision events (Fig. 1a). In this configuration, which we call *Brainbow-1*, two recombination events are initially possible, but only one can occur. Excision between either pair of identical *lox* sites removes one of the other pair, thereby preventing further recombination.

Brainbow-1.0. We first verified that the *lox* variant *lox2272* (ref. 12 and Supplementary Table 1), which is incompatible with *loxP* in prokaryotic systems, would be comparably selective in mammalian cells (Supplementary Fig. 1a, b). We then built a construct, *Brainbow-1.0*, in which the arrangement of *loxP* and *lox2272* allows two possible recombination outcomes, switching XFP expression from default red to either yellow or cyan (Fig. 1a). As expected, HEK 293 cells stably transfected with the construct expressed only red fluorescent protein (RFP) from the cDNA immediately adjacent to the promoter. On Cre action, many cells lost RFP expression and switched on YFP or CFP, creating a three-colour cellular array (Fig. 1b). Yellow and cyan cells were present in comparable numbers (50 ± 15 YFP^+ cells and 52 ± 12 CFP^+ cells per field), suggesting that neither recombination pathway is strongly favoured over the other. Co-expression of YFP and CFP was not observed in the cell line shown in Fig. 1b, but was seen in other clones, presumably because of the presence of multiple copies of the transgene (see below).

Brainbow-1.1. To permit expression of a fourth XFP, we designed an additional *lox* variant, the efficiency of which was equivalent to *loxP*

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and *lox2272* but was incompatible with both of them (Supplementary Fig. 1c, d). This new variant, *loxN*, has substitutions in the *lox* spacer that differ from both *loxP* and *lox2272* (Supplementary Table 1). Using *loxN*, we built a *Brainbow-1.1* construct bearing orange (OFP), red, yellow and cyan fluorescent protein genes, where Cre is offered a choice between three recombination events (Fig. 1c). In the absence of recombination, HEK cell lines expressing this construct showed OFP fluorescence, whereas Cre triggered expression of the other XFPs (Fig. 1d).

Stochastic recombination using Cre-mediated inversion. Cre can invert DNA segments flanked by *loxP* sites facing each other. Inversion can continue for as long as the recombinase is present, but restricting Cre activity to a limited time stabilizes the transgene in one or the other orientation independently in each cell[11]. In *Brainbow-2*, we use inversion to obtain multiple XFP expression.

Brainbow-2.0. We first built a Cre-invertible construct containing *RFP* and *CFP* in head-to-head orientation (Fig. 2a). In HEK cells, this construct, *Brainbow-2.0*, drove expression of the sense-oriented *RFP*. Transient transfection with Cre activated expression of the antisense-oriented *CFP* in some cells, indicating that the inversion strategy was effective (Fig. 2b).

Brainbow-2.1. On the basis of the success of *Brainbow-2.0*, we designed *Brainbow-2.1*, in which two invertible DNA segments are positioned in tandem to generate a larger number of recombination outcomes (Fig. 2c). Three different inversions can occur in *Brainbow-2.1* (see i, ii and iii in Fig. 2c). In addition, either of two excision events (iv, v) may reduce the construct to one of two single invertible DNA segments, which can continue to invert as long as Cre is present. These different recombination possibilities can be used to express four genes. HEK cells stably transfected with a *Brainbow-2.1* construct containing green, yellow, red and cyan fluorescent protein showed mosaic expression of all XFPs after transient transfection with Cre (Fig. 2d).

Thus, stochastic expression of up to four XFPs can be obtained from a single *Brainbow-1* or *Brainbow-2* transgene in which the Cre recombinase is offered a choice of excision and/or inversion between multiple pairs of *lox* sites.

Brainbow *in vivo*

***Thy1-Brainbow* transgenic mice.** To generate lines of mice expressing *Brainbow* in neurons, the *Brainbow-1* and *Brainbow-2* constructs were placed under the control of regulatory elements from the *Thy1* gene, which are known to drive expression at high levels in a variety of neuron types[7,13]. Nineteen *Thy1-Brainbow* lines showing transgene expression were obtained: twelve with the *Brainbow-1.0* construct, one with *Brainbow-1.1*, two with *Brainbow-2.0* and four with *Brainbow-2.1* (Supplementary Table 2). The labelled cell types were similar to those seen previously in *Thy1* transgenic animals[7,13], although expression in glial cells was also observed here in four lines (see below).

To induce recombination in the *Thy1-Brainbow* mouse lines, we crossed them with *CAGGS-CreERT2* animals[14]. In these mice, *CAGGS* drives broad expression of an oestrogen receptor–Cre fusion specifically activated by injection of the ligand tamoxifen[15]. We analysed double heterozygous adult animals (*CAGGS-CreERT2*$^{+/-}$; *Thy1-Brainbow*$^{+/-}$) that had been injected with a single dose of tamoxifen at postnatal day 0–3 (P0–P3) to activate recombination in cells co-expressing *Brainbow* and CreER. Recombination leading to new colours was observed in all 19 *Brainbow* lines (Fig. 3a–d). In most lines, the number and location of expressing cells appeared unchanged after recombination. However, in some lines the population of cells expressing XFPs was larger in the recombined animals than in the non-recombined controls (Supplementary Table 2). This

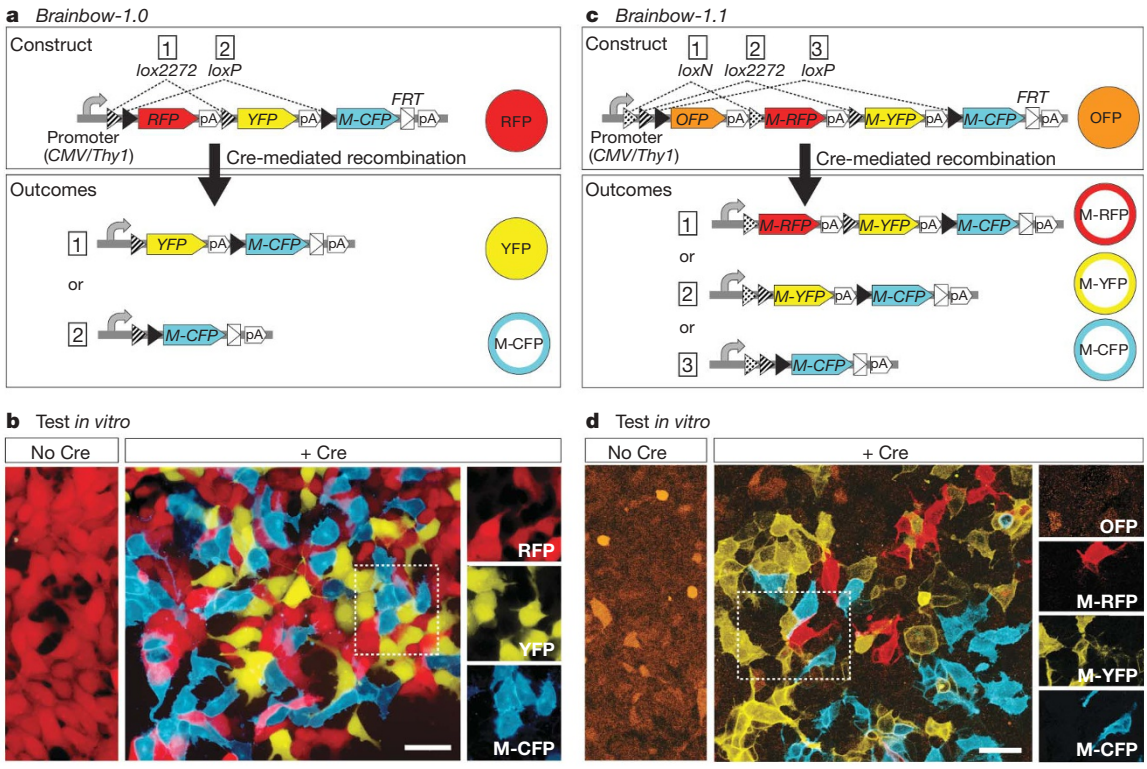

Figure 1 | *Brainbow-1*: stochastic recombination using incompatible *lox* variants. **a**, In *Brainbow-1.0*, incompatible sets of *lox* sites alternate: Cre chooses between excision events 1 or 2. Before Cre action, only the gene following the promoter is expressed (RFP). Recombination switches expression to either YFP (1) or M-CFP (2). **b**, HEK cells stably transfected with *CMV-Brainbow-1.0* express RFP. On transient transfection with Cre, these cells randomly switch to YFP or M-CFP expression. **c**, In *Brainbow-1.1*, a third set of incompatible *lox* sites (*loxN*) is added, creating three recombination possibilities (1, 2 or 3), switching OFP expression to RFP, YFP or CFP expression. **d**, Cells stably transfected with *Brainbow-1.1* express OFP. Cre recombination leads to expression of M-RFP, M-YFP or M-CFP. pA, polyadenylation signal; M-XFP, membrane-tethered XFP. *FRT* site allows reduction of transgene arrays (Fig. 4d). Scale bar, 50 μm.

altered expression is consistent with the observation that shortening a transgene can increase its probability of expression[16]. These tests show that recombination of both *Brainbow-1* and *Brainbow-2* transgenes leads to mosaic expression of multiple fluorescent proteins *in vivo*. As was the case *in vitro*, no XFP expression other than the default was detected in the absence of Cre (Supplementary Fig. 3a).

Combinatorial XFP expression. In some *Thy1-Brainbow* mouse lines, individual neurons expressed only one of the XFPs present in the transgene (Fig. 3d and Supplementary Table 2). More commonly however, transgenic *Brainbow* lines showed co-expression of multiple colours in individual cells (Figs 4 and 5, Supplementary Fig. 2 and Supplementary Table 2). Notably, a large number of different hues was observed. In a confocal stack through a peripheral motor nerve, for instance, we distinguished at least ten XFP combinations in different axons (Fig. 4b). Neurons of the central nervous system also exhibited combinatorial XFP expression (Figs 4c and 5, and Supplementary Fig. 2).

How were these colour combinations generated? Polychromatic cells were not unexpected given that pronuclear injection typically leads to the tandem integration of multiple transgene copies[17]. If, for example, three copies of the *Brainbow-1.0* construct were present in mice and recombined independently, at least ten colour mixtures would be expected (Fig. 4a). Indeed, quantitative polymerase chain reaction (PCR) tests detected eight transgene copies in *Brainbow-1.0* line H and 16 in line G (data not shown). To investigate whether combinatorial XFP expression was caused by these multiple copies, we reduced their number[16], making use of an *FRT* site that we had inserted into the *Brainbow* transgene (Fig. 1a). Applying Flp/*FRT*-mediated recombination (analogous to the Cre/*lox* system but using a different recombinase and target sequence[10]), we excised tandem repeats of the *Brainbow* construct by crossing lines G and H with Flp-expressing mice[18] (Fig. 4d). Two *Thy1-Brainbow-1.0*$^{(Flp)}$ lines were

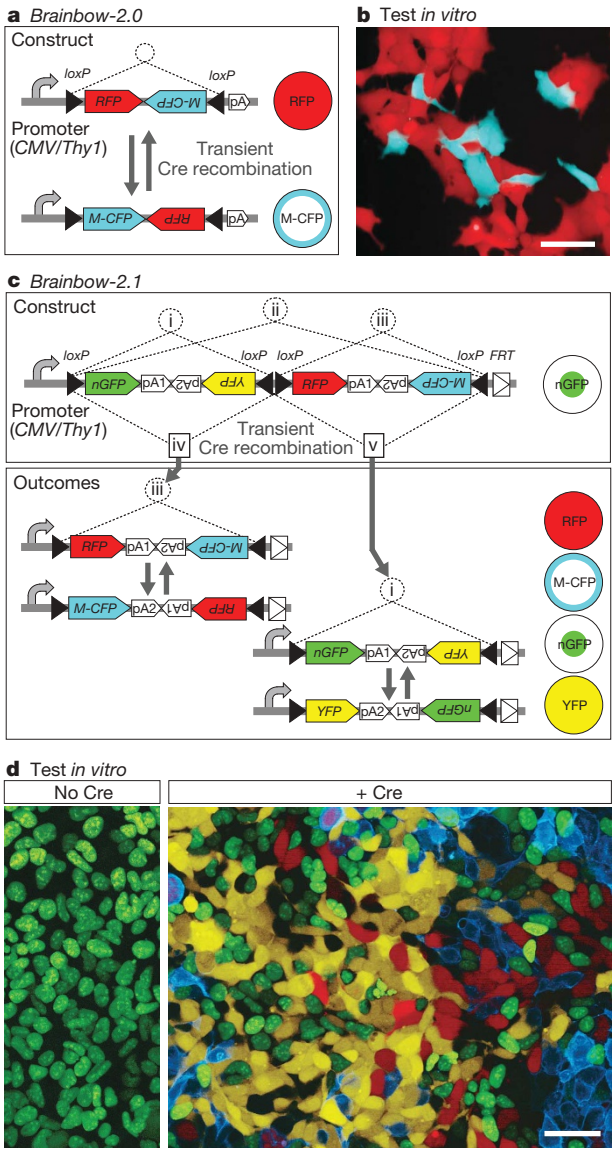

Figure 2 | *Brainbow-2*: stochastic recombination using Cre-mediated inversion. **a**, In *Brainbow-2.0*, Cre triggers inversion of a DNA segment flanked by *loxP* sites in opposite orientation. In 50% of cells, inversion should end in an antisense orientation and switch gene expression. **b**, HEK cells stably expressing *CMV-Brainbow-2.0* produce RFP, and stochastically switch to CFP expression when transfected with Cre. **c**, The *Brainbow-2.1* construct contains two tandem invertible DNA segments. Inversion (i–iii) and excision (iv, v) recombination events create four expression possibilities. **d**, Stable *CMV-Brainbow-2.1* transfectants express nuclear GFP (nGFP). Cre recombination triggers expression of YFP, RFP or M-CFP. pA1 and pA2, SV40 and bGH polyadenylation signals. Scale bars, 50 μm.

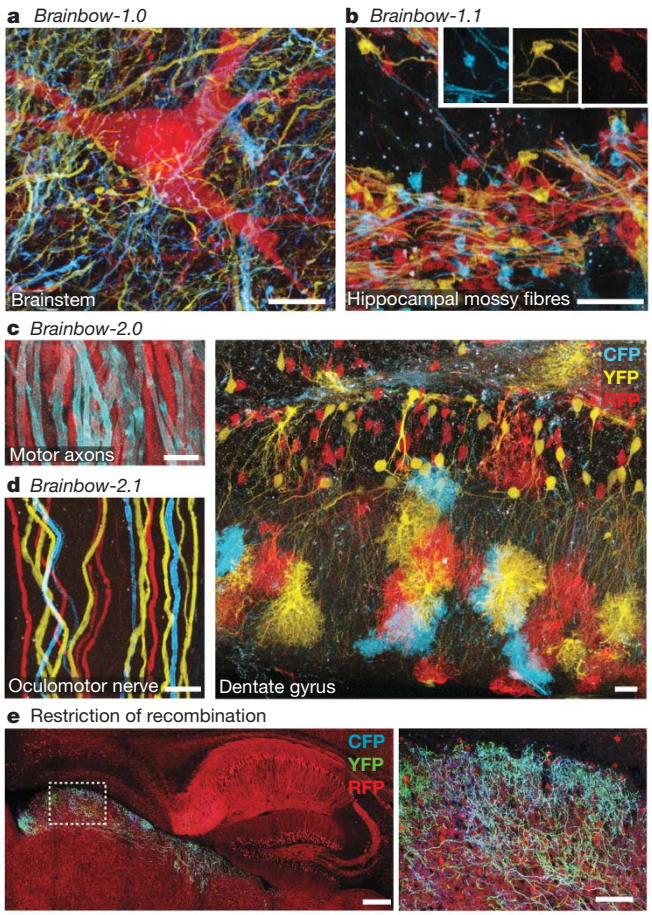

Figure 3 | XFP expression in *Brainbow* transgenic mice. **a**, **b**, *Thy1-Brainbow-1.0* and *Thy1-Brainbow-1.1* transgenic mice were crossed with CreERT2-expressing animals. Tamoxifen injection led to mosaic XFP expression throughout the brain. **a**, Brainstem, line H; **b**, hippocampal mossy fibre axons and their terminals (see insets), line M. **c**, In *Thy1-Brainbow-2.0* mice, transient recombination with the CreERT2/tamoxifen system triggers expression of M-CFP (peripheral motor axons, line N). **d**, In *Thy1-Brainbow-2.1* mice, CreERT2-mediated recombination leads to expression of multiple XFPs. Left: oculomotor nerve, line R. Right: hippocampus (dentate gyrus), line Q (labelled neurons and astrocytes). **e**, Sagittal brain sections of *Thy1-Brainbow-1.0* mice line H crossed with the retina-specific *Chx10-Cre* driver. Recombination is almost completely restricted to retinal ganglion cells, as shown by label of their axons arborizing in the superior colliculus. The boxed area in the left panel is shown at greater magnification in the right panel. Scale bars: **a**–**c**, 10 μm; **d**, 20 μm; **e** (left), 250 μm; **e** (right), 50 μm.

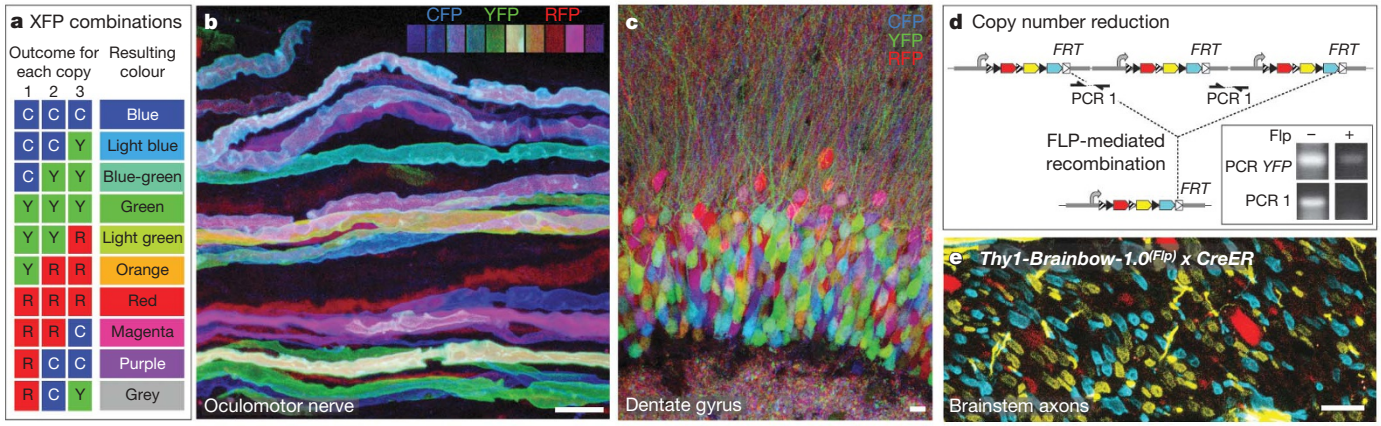

Figure 4 | Combinatorial XFP expression results from tandem copy integration. **a**, With a *Brainbow* construct expressing three XFPs, independent recombination of three transgene copies can, in principle, generate ten distinct colour combinations. **b**, Oculomotor axons of *Thy1-Brainbow-1.0* line H (recombination with CreERT2). Boxes show sample regions from different axons. **c**, Dentate gyrus of *Thy1-Brainbow-1.0* line L (recombination with CreERT2). **d**, A single *FRT* site inserted in *Brainbow* constructs allows tandem transgene copy number reduction through Flp-mediated recombination. The PCR indicates the disappearance of transgene repeats in *Thy1-Brainbow-1.0* line H crossed with Flp-expressing mice (inset). **e**, A Flp-recombined line derived from line H expresses XFPs in a mutually exclusive manner. Scale bars, 10 μm.

derived that showed mutually exclusive rather than combinatorial XFP expression in all animals tested ($n = 5$ for each line, Fig. 4e).

Thus, combinatorial gene expression in *Brainbow* mice appears to be generated by multiple copies of the transgene recombining independently towards different outcomes. Partial inter-copy recombination might also contribute to the palette of colour observed.

Recombination with specific Cre drivers. In principle, stochastic expression from the excision-based *Brainbow-1* transgene could be activated by chronically active Cre rather than via tamoxifen-inducible CreER. To test this idea, we crossed *Thy1-Brainbow-1.0* mice showing neuronal expression to *Chx10* (also called *Vsx2*)-*Cre* mice, which drive recombination in the retina[19]. *Brainbow* expression

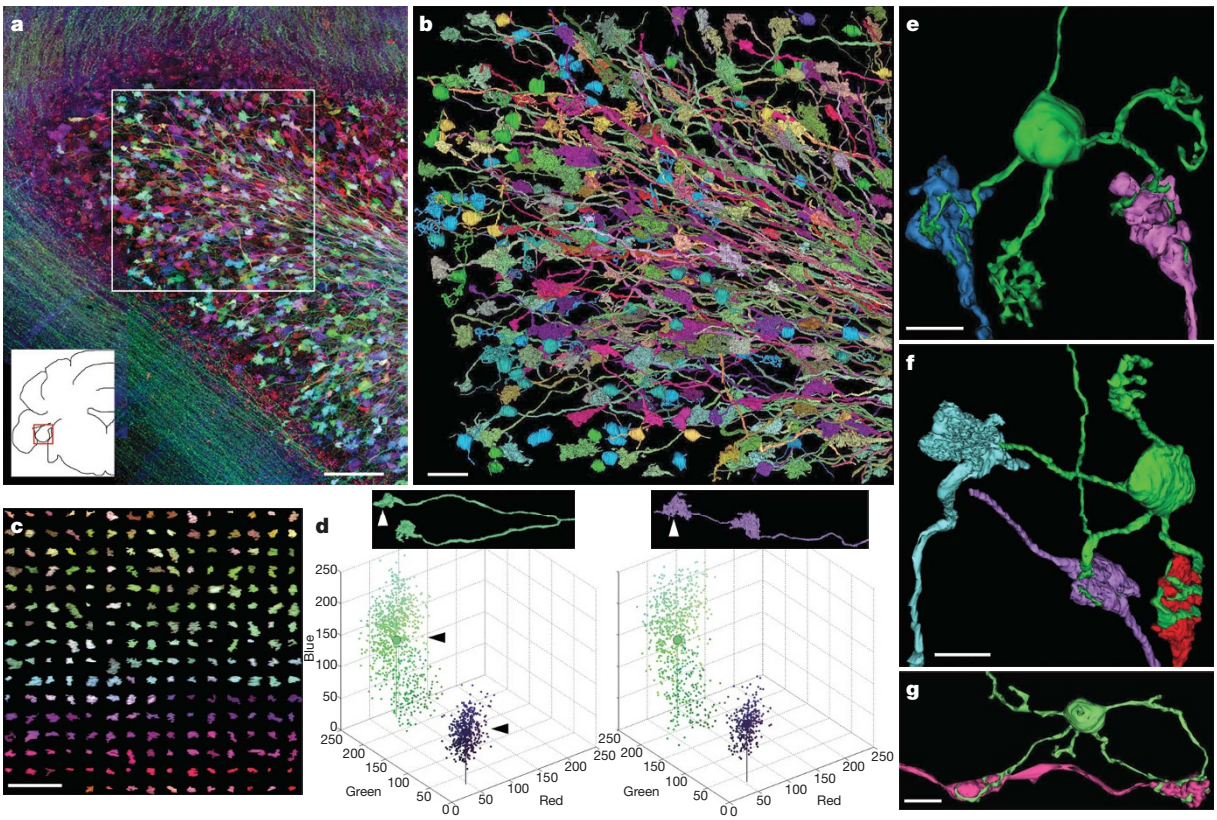

Figure 5 | Cerebellar circuit tracing and colour analysis. **a**, Cerebellar flocculus from line H. Inset shows coronal location. **b**, Three-dimensional digital reconstruction of region boxed in **a** (341 axons and 93 granule cells; 160 μm^2 × 65 μm). (See also Supplementary Movie 1.) **c**, Colour distribution of rosettes. Each rosette from the reconstructed region is aligned according to hue. **d**, Colour constancy along axon. Top: two mossy fibre axons (green, purple) each possess two presynaptic rosettes. Bottom: pixel distribution for each rosette (R, G and B intensities displayed on a scale from 1 to 255). The left graph displays upper rosettes for each axon (arrowheads); right graph displays corresponding lower rosette. **e**, Reconstructed granule cell receives input from ≥3 different mossy fibres (blue, pink and at least 1 unlabelled). The granule cell axon projects upwards. **f**, Each granule cell dendrite is innervated by a different presynaptic neuron (three labelled, one unlabelled). **g**, Two granule cell dendrites are innervated by the same presynaptic mossy fibre. Scale bars: **a**, **c**, 50 μm; **b**, 15 μm; **e**–**g**, 5 μm.

in the brain (YFP, CFP and combinations) was restricted to retinal projections (Fig. 3e). Additional Cre lines tested similarly drove *Brainbow* expression in territories where expression of *Thy1* and Cre intersected (Supplementary Fig. 3).

Circuit tracing with Brainbow

To determine whether combinatorial XFP expression in *Brainbow* mice can be used as a tool for mapping neural circuits, we studied the inner granular layer (IGL) of the cerebellum. *Brainbow-1.0* line H labelled two interconnected components of IGL circuitry: subsets of mossy fibre axons and their postsynaptic targets, granule cells. Quantitative features of this circuit, such as the number of presynaptic neurons that contact a postsynaptic granule cell, have thus far been largely inaccessible. Although it is often assumed that granule cells integrate signals from converging inputs arising from different cells[20], this has not been shown directly. Multiple presynaptic terminals of one mossy fibre axon are sometimes densely clustered in small regions of the IGL[21], raising the possibility that a single mossy fibre provides multiple inputs to the same granule cell.

Reconstruction. We used confocal microscopy to image the cerebellar flocculus from an adult (P79) *CAGGS-CreERT2*$^{+/-}$; *Thy1-Brainbow-1.0*$^{+/-}$ mouse (line H) treated with tamoxifen at P0 (Fig. 5a). Spectrally non-overlapping signals from each of the three XFPs were collected into separate channels (R, G and B). We observed a wide range of colour combinations in different axons, allowing us to distinguish neighbouring axons and trace them through the volume by computer-assisted methods (Fig. 5b and Supplementary Movie 1). The colour information markedly facilitated the tracing process (Supplementary Fig. 4). In total, we reconstructed 341 axons and 93 granule cells. These data included 236 mossy fibre presynaptic terminals or 'rosettes' (Fig. 5c).

Potentially, colour might be used to verify the identity of all the processes arising from an individual neuron without necessarily tracing back to proximal branch points or the soma. This approach would require that the colour profile of a neuronal process remain constant over long distances. In order to study colour constancy, we sampled consecutive mossy fibre rosettes along individual axons and compared their RGB values (Supplementary Fig. 5a, b). The colour profiles obtained for distant regions of a mossy fibre axon (rosettes more than 100 μm apart along a given axon) were largely similar (Fig. 5d). Moreover, axons and dendrites belonging to the same neuron also exhibited similar colour profiles (Supplementary Fig. 6).

Given the colour constancy within a cell, colour differences provide a way to distinguish between neurons and thus could be useful for detailed circuit analysis, such as to count the number of neurons that innervate a postsynaptic cell. Our analysis of cerebellum revealed numerous synaptic interactions between mossy fibres and granule cell dendrites, identified by their characteristic claw-like morphology[22]. We found that individual granule cells were typically innervated by multiple axons that expressed different colours (Fig. 5e, f). Hence, more than one presynaptic neuron innervated each postsynaptic neuron. In several instances, each dendrite of a single granule cell was unambiguously innervated by a different presynaptic neuron (Fig. 5f). In one case, a granule cell was contacted by two presynaptic terminals from a single mossy fibre (Fig. 5g), but it also received inputs from at least one additional (unlabelled) mossy fibre. These data are consistent with the idea that cerebellar granule cells are polyneuronally innervated by mossy fibres.

How many colours? The usefulness of the Brainbow system to analyse complex connectivity depends on the number of distinguishable colours expressed by neurons. To determine this number, we analysed the distribution of colour profiles in the reconstructed volume from line H above (eight transgene copies). The population of axons exhibited many different colour profiles (Fig. 5c); the mean colour values calculated for the different axons varied greatly in hue and saturation and filled a large portion of colour space (Supplementary Fig. 5c). Using a visual colour discrimination test, we found that 98.9% of randomly selected rosette pairs expressed colours distinct enough to discriminate (see Methods). This degree of colour variation is equivalent to having approximately 89 distinct colours (that is, if 98.9% of axon pairs appear different, then the remaining 1.1% or 1 out of 88.7 pairs are too similar to discriminate). An alternative computer-based colour analysis of hippocampal neuron cell bodies from *Brainbow-1.0* line L (see Fig. 4c) gave an estimated 166 colours. This large number of colours should be useful in resolving individual components of many neural circuits.

Brainbow analysis of glial interactions

The Brainbow strategy can be used to reveal interactions among neighbouring non-neuronal cells as well as neurons. Glial cells were labelled in four of the *Thy1-Brainbow* lines that we generated (Fig. 6). Two lines showed transgene expression in large populations of astrocytes (Fig. 6a, b; see also Fig. 3d), a third in cerebellar Bergmann glia (Fig. 6c, d) and a fourth in non-myelinating Schwann cells, including those at neuromuscular junctions (Fig. 6e, f).

Tiling. The anatomical relations among glial cells and their interactions with neurons have been studied extensively[23,24]. Brainbow provides a new method for approaching this issue. Recombination of *Thy1-Brainbow-1.1* line M led to mutually exclusive expression of three membrane-targeted XFPs in astrocytes throughout the brain, providing unambiguous delineation of boundaries between adjacent astrocytes (Fig. 6a). Single confocal sections showed that adjacent astrocytes were interdigitated in a complex way at their periphery (Fig. 6b). However, each astrocyte possessed a core contiguous territory where it was the only astrocyte present, supporting previous conclusions[23]. Such an exclusive arrangement between individual glial cells and a region of neuropil was not found for a second type of astrocyte labelled in *Thy1-Brainbow-1.0* line G: cerebellar Bergmann glial cells. The thin glial sheath made by Bergmann glia around Purkinje cell dendrites showed extensive interdigitation, with multiple glial cells ensheathing a single portion of dendrite (Fig. 6c, d).

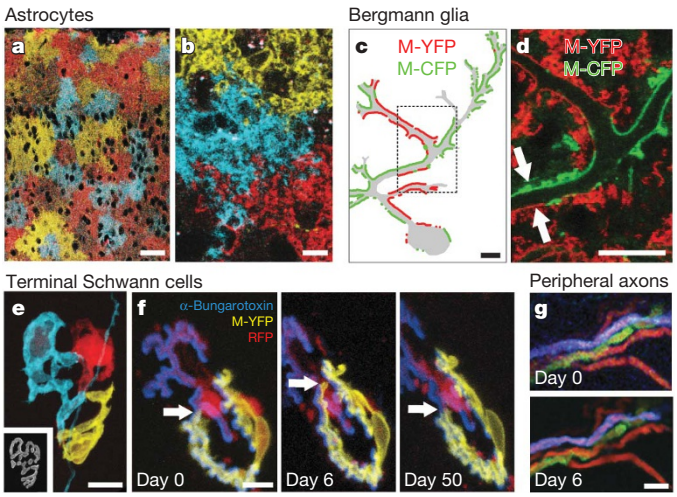

Figure 6 | *Brainbow* expression in glial cells and time-lapse imaging. **a**, **b**, *Thy1-Brainbow-1.1* line M: astrocytes labelled with three different M-XFPs tile the brain (cortex, **a**) and interdigitate (colliculus, **b**). **c**, **d**, *Thy1-Brainbow-1.0*$^{(Flp)}$ line G: M-YFP and M-CFP (shown as red and green) are expressed in a mutually exclusive manner in Bergmann glial cells of the cerebellum (RFP channel not shown). Reconstruction of a Purkinje cell (grey) reveals tiled Bergmann glial cells associated with different parts of its dendritic tree (**c**) and tightly apposed (**d**, arrows). **e**, *Thy1-Brainbow-1.0* line B: three non-myelinating Schwann cells (blue, yellow, red) tile one neuromuscular junction (inset: cholinergic receptors labelled with Alexa647-bungarotoxin). **f**, Time-lapse images show changes in the territory of individual Schwann cells over 50 days. The cell labelled with M-YFP extends and then retracts a process (arrow). **g**, Time-lapse images of a neuromuscular nerve. The colour of individual axons remained constant over the period imaged. Scale bars: **a**, 50 μm; **b**–**g**, 10 μm.

Thus, Bergmann glial cells do not appear to occupy domains related to specific parts of neurons, nor to have exclusive control of an uninterrupted territory.

Time-lapse imaging

At the adult neuromuscular junction in *Thy1-Brainbow-1.0* line B, it was sometimes possible to visualize each of the individual terminal Schwann cells (Fig. 6e). We performed time-lapse studies on these cells. In 15 out of 17 junctions (in seven mice) imaged over intervals of 4–50 days, we found that the boundaries between adjacent glial cells ensheathing the same motor axon terminal shifted with time, sometimes alternating back and forth (Fig. 6f).

In long-term time-lapse studies of axons that involve repetitive imaging, Brainbow might be useful to identify a specific neuron in successive imaging sessions, assuming a neuron retains its colour. To explore this possibility we imaged the same region of a neuromuscular nerve over 6 days and found that *Brainbow* expression in individual axons remained constant over time both in terms of colour and intensity (Fig. 6g). Thus, the colour tags provided by *Brainbow* are stable and expressed at sufficient levels to permit longitudinal studies of circuits *in vivo*.

Discussion

Brainbow transgenes are novel reagents for large-scale studies of cellular interactions. These Cre/*lox*-based transgenes create a mosaic gene expression offering two key advantages. First, they use DNA excision and/or inversion to create a stochastic choice among several genes—up to four with the configurations presented here as opposed to two with other methods[9–11]. Notably, no specific pairs of *lox* sites or transgene orientations are strongly favoured, so distinct outcomes can occur at similar frequencies. Moreover, following recombination the expression choice is stabilized, either by using *lox* variants which prevent further excision in *Brainbow-1*, or by transient Cre action (permitted by several strategies[11,15,25]) to stop inversion in *Brainbow-2*. Second, this stochastic choice can give rise to either mutually exclusive gene expression when a single copy of the construct is present, or to combinatorial expression when there are multiple copies. Several parameters might affect the diversity of the combinations obtained, including promoter choice, transgene copy number and length, efficiency and duration of the recombination.

In the constructs presented here, combinatorial expression of three XFP genes generates approximately 100 colours in neurons. This labelling appears well suited for visualization and tracing of large numbers of neurons and their connectivity. We show that colour differences between neurons provide a way to sort their processes while tracing through sections, to directly visualize their putative synaptic interactions, and to distinguish the neurons that converge onto a postsynaptic cell. The power of this circuit analysis is presently limited by the number and distribution of colours and the ability to resolve them; the lack of a concomitant way to identify unambiguously synaptic contacts; and the restriction of *Thy1* expression to certain neuronal populations. Future constructs could address most of these limitations by taking advantage of the ever-expanding spectrum of fluorescent proteins[5], promoters[26,27] and Cre drivers[10] as well as by combining Brainbow with transgenic synaptic markers or ultrathin sectioning techniques[3].

Brainbow's ability to switch on multiple genes may also prove useful for other purposes. The *Brainbow* cassettes could be adapted to any cell type and to any organism in which site-specific recombinases act. We anticipate that Brainbow could be used in lineage analysis to mark neighbouring clones with distinct colours. Brainbow will also be useful to visualize individual cells and their interactions, as demonstrated here for both neurons and glial cells. Finally, this approach raises tantalizing possibilities for modulating gene expression and comparing the effects of several distinct genetic changes (or their combination) simultaneously introduced into a population of cells.

METHODS SUMMARY

Brainbow constructs were assembled by standard cloning methods using the following XFPs: mEYFP (monomeric EYFP[28]), mCerulean[29], Kusabira orange[30], tdimer2(12)[31], mCherry[32], dTomato[32], ECFP and dsRed2 (Clontech), and hrGFPII-NLS (Stratagene). A palmitoylation sequence[33] tethered XFPs to the membrane in some constructs, allowing clear labelling of axonal processes, whereas cytoplasmic XFPs better labelled neuronal cell bodies and dendrites. A novel *lox* variant, *loxN*, was created with mutations distinct from both *loxP* and *lox2272*, making all three *lox* sites mutually incompatible. For *in vitro* tests, HEK cells stably expressing *CMV-Brainbow* constructs were selected. Recombination was induced by transient *CMV-Cre* transfection. For transgenic mouse generation, *Brainbow* constructs were cloned in a *Thy1.2* cassette[13]. Three to nine independent lines were obtained for each construct and crossed with *CAGGS-CreERT2* mice[14]. Their offspring received a single injection of 10–50 μg tamoxifen between P0 and P3 (ref. 34). For transgene copy reduction, mice were crossed with *β-actin-Flpe* animals[18]. Anaesthetized mice were perfused with 2–4% PFA. Brain sections, whole-mount skeletal muscle, nerves and fixed HEK cells were imaged using a Zeiss Imager.Z1 epifluorescence microscope (with standard CFP, YFP, TRITC and TxRed filters), or an Olympus FV1000 confocal microscope (equipped with 440-, 515- and 568-nm lasers). Live imaging methods were adapted from ref. 35. In all lines XFP fluorescence was directly observable, except for *Brainbow-2.0* animals, which required anti-GFP immunostaining. Linear unmixing was used only for the constructs expressing overlapping dyes (OFP/RFP and CFP/GFP/YFP). Tracing and three-dimensional reconstruction were performed using Reconstruct software[36]. Colour profiles of axon sections were analysed and displayed using Matlab. Visual- and computer-based colour quantifications are described in Methods.

Four of the most generally useful *Thy1-Brainbow* mouse lines described here (H, L, M and R) are available to the academic research community through Jackson Laboratory (JAX Stock Numbers 007901, 007910, 007911 and 007921, respectively).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions J.L., J.R.S. and J.W.L. conceived the Brainbow strategies. J.R.S. and J.W.L. supervised the project. J.L. built initial constructs and validated them *in vitro* and *in vivo*. T.A.W. performed all cerebellar axonal tracing and colour profile analysis with programs developed with J. Lu. H.K. performed all live imaging experiments. R.W.D. generated *Brainbow-1.0* lines expressing cytoplasmic XFPs, and R.A.B. generated *Brainbow-1.1* constructs and lines. J.L., T.A.W. and R.W.D. screened mouse lines.

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METHODS

DNA constructs. *Brainbow* constructs were assembled by standard cloning methods, using the CMV expression vector pEYFP-N1 (Clontech) as a start point. Briefly, annealed oligonucleotides were used to insert *lox* sites in pEYFP-N1 to generate *lox-EYFP*. Other fluorescent proteins were then cloned in place of *EYFP*, and these *lox-XFP* units were ligated stepwise to generate the *CMV-Brainbow* constructs used for *in vitro* tests. For the *Brainbow-2.1* construct, a nuclear localization signal (NLS) was added to the carboxy-terminal end of hrGFPII to permit unambiguous distinction from its close spectral neighbours CFP and YFP. For the *Brainbow-1.1* construct, a novel *lox* variant, *loxN*, was created with substitutions in position 2 and 7 of the *lox* spacer that differed from both *loxP* and *lox2272*, making all three *lox* sites mutually incompatible (Supplementary Table 1). An *FRT* site was inserted at the 3′ end of each *Brainbow* construct to permit reduction of transgene multicopy by action of Flp recombinase (Fig. 4d and ref. 16). *Brainbow* cassettes were subsequently cloned in the *Xho*I site of a *Thy1.2* genomic element[13]. Except in *Brainbow-2.0*, each reading frame was followed by a polyadenylation signal (SV40, bGH, or from the 3′ part of the *Thy1* element) to maximize expression[37].

Cell culture. *CMV-Brainbow* constructs bearing a *neo*^{r} selection marker were linearized and electroporated into HEK cells. Stable transfectants were selected with G418 and fluorescent clones were picked and expanded. Recombination was induced in these cells by transient transfection with a *CMV-Cre* vector using Lipofectamine 2000 (Invitrogen). After 3–12 days *in vitro*, cells were fixed for 10 min with cold 4% PFA, and samples were mounted in Vectashield before imaging.

Mice. *Thy1-Brainbow* constructs were linearized with *Eco*RI and *Pvu*I and injected into pronuclei to generate 3–9 independent transgenic lines per construct. Genotyping and transgene copy number estimation were performed with PCR using YFP primers. Lines were crossed with *CAGGS- CreERT2* mice[14]. Their offspring received a single dorsal subcutaneous injection of 10–50 μg tamoxifen (Sigma T5648, 1–5 mg ml^{-1} in corn oil) between P0 and P3, in order to induce widespread recombination throughout the nervous system[34]. *Thy1-Brainbow-1.0* lines F and H were also crossed to four Cre driver lines known to trigger recombination in specific nervous system regions: *Islet1*^{Cre} in retina and basal ganglia[38], *Chx10-Cre* and *Pax6*$_{\alpha}$*-Cre* in retina[19,39], and *Emx1*^{Cre} in cortex[40]. In each case, we observed *Brainbow* expression (YFP, CFP and combinations) in the territories where expression of *Thy1* and the Cre driver intersect (Fig. 3e; Supplementary Fig. 3 and data not shown). For germline Flp-induced transgene copy reduction, mice were mated to *β-actin-Flpe* animals[18]. Interestingly, transgene copy reduction of *Brainbow-1.0* line H by Flp/*FRT* necessitated three generations before a line showing mutually exclusive XFP expression was derived. This may be related to the large size of the construct (10 kb for *Thy1-Brainbow-1.0*), as site-specific recombination is known to be affected by distance[41,42]. To verify transgene copy number reduction, we used a PCR spanning the junction between adjacent transgene copies.

Four of the most generally useful *Thy1-Brainbow* mouse lines described here (H, L, M and R) are available to the academic research community through Jackson Laboratory (JAX Stock 007901, 007910, 007911 and 007921, respectively).

Tissue preparation. Animals between P8 and P40 were anaesthetized with sodium pentobarbital before intracardiac perfusion with PBS and 4% PFA. One-hundred-micrometre sagittal brain sections were obtained using a vibrating microtome (Leica VT1000). Sections and fixed tissues were mounted in Vectashield and stored at −20 °C before imaging. In the case of *Brainbow-2.0* mice, which possessed the only construct that did not include polyadenylation signals directly after each XFP, the CFP signal was amplified with an anti-GFP antibody (Chemicon Ab3080).

Imaging. Fixed brain and muscle samples were imaged with ×20 (0.8 NA), ×40 (1.3 NA) or ×60 (1.45 NA) oil objectives. Confocal images were acquired with an Olympus FV1000 microscope equipped with a 440/515/568/633-nm primary beam splitter, using a 440-nm photodiode laser for CFP (PMT1, 510-nm dichroic mirror, 465–495-nm barrier filter), a 515-nm argon line for YFP (PMT2, 560-nm dichroic mirror, 530–565-nm barrier filter), and a 561-nm photodiode laser or 568-nm krypton laser for RFP (PMT3, 585–615-nm barrier filter). Confocal image stacks for all three channels were acquired sequentially, and maximally projected using Metamorph or ImageJ software. For cell culture experiments, linear unmixing was performed with Olympus or ImageJ software on single-plane confocal images obtained on the setup described above to separate OFP/RFP signals in *Brainbow-1.1* and CFP/GFP/YFP signals in *Brainbow-2.0*, using a 10-nm collection window and 10-nm step for the lambda scan (in some cases linear unmixing was performed on epifluorescence images of cell cultures).

In Adobe Photoshop, the 12-bit images obtained for each channel were imported in the 16-bit mode and then mapped to an 8-bit display before uniform adjustment of levels, contrast, brightness and gamma. The channels were then assigned colours and overlaid. Unless noted, CFP, YFP and RFP fluorescence was displayed using cyan, yellow and red colour (in some cases using blue, green and red).

Tracing and three-dimensional reconstruction. We used Reconstruct, a program to assist three-dimensional reconstruction from serial sections available at http://synapses.bu.edu[36]. Confocal data stacks were loaded as RGB tiffs in either the XY or YZ plane. Tracing of cross-sections was done using the automated 'wildfire' tool where possible, otherwise by hand.

Colour analysis. We used Matlab software to measure colour in each traced section throughout the data set, then calculated the pixel distribution and centroid (or mean colour) for each reconstructed axon. The Matlab rgb2hsv function was used to translate centroid colours from RGB to HSV coordinates. In the visual colour-discrimination test (done on mossy fibre rosettes from *Brainbow-1.0* line H), we compared pairs of images randomly selected from the 236 reconstructed mossy fibre rosettes ($n = 6{,}225$). Matlab presented random samples from two rosettes on the computer screen and an observer determined whether the samples were drawn from the same or different rosettes. In the computer-based analysis (done on dentate gyrus granule cells from *Brainbow-1.0* line L), a colour criterion was calculated (eight units in RGB space) based on a within-cell analysis that compared the colour centroids of two regions within each cell soma. This criterion was then used for a between-cell comparison that searched all the other imaged neurons (>200) to determine the total number of distinct colours present in the data set. The result showed that there were 166 different colours (128 neurons expressed unique colours, 52 were indistinguishable from one other cell, 20 were indistinguishable from two other cells and two were indistinguishable from three other cells).

Live imaging. Methods for repetitive vital imaging of 1–3-month-old mice were adapted from ref. 35. Acetylcholine receptors were labelled by application of Alexa-647-conjugated α-bungarotoxin (Molecular Probes) in sterile, lactated Ringer's solution. We used the FV1000 confocal setup described above equipped with a ×20, 1.0 NA water immersion objective. All channels were acquired simultaneously.

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