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Matching neural morphology to molecular expression: Single cell injection following immunostaining

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Abstract

To match a neuron's morphology with its expression of a particular protein, it is useful to first identify the cell by immunostaining and then inject it with fluorescent dye. Such targeted injection cannot be performed with a hydrophilic dye (such as Lucifer yellow) because the neuron, once rendered porous to antibodies, does not retain it. But a lipophilic dye (such as DiI) injected iontophoretically into the soma forms a crystal and is thereby trapped. From this intracellular depot dye diffuses into the cell membrane to reveal the detailed morphology. We have used this strategy to identify the morphology of a GABAergic retinal bipolar cell and several types of GABAergic amacrine cell. In addition, we demonstrate probable connections from a narrow-field, GABAergic amacrine cell to the OFF brisk-transient ganglion cell. Finally, we show that the strategy works in the cortical slice, showing a layer IV cell immunostained for parvalbumin to be a "nest basket cell".

Introduction

A complex neural tissue, such as retina or cortex, comprises diverse cell types, each defined by specific protein expression and morphology. If a protein is expressed restrictively, that is by only a few cell types, and if it is expressed strongly throughout a cell, simple immunostaining can identify both the molecule and the cell's morphology. However, many important proteins are expressed too weakly or too locally within the cell to reveal its morphology. And commonly a protein is expressed by multiple cell types whose different morphologies cannot be easily disentangled by immunostaining. This forces one to separately investigate protein expression and morphology, and try somehow to draw the two categories into correspondence.

For morphology, neurons are often injected with a hydrophilic compound, such as Lucifer yellow, in live tissue and then fixed and immunostained. But where cell types are diverse, a neuron chosen at random for filling is unlikely to express the molecule of interest. For example, a small subset of bipolar neurons in cat retina expresses the GABA-synthetic enzyme, GAD_{65} (Vardi & Auerbach, 1995). But bipolar neurons in cat retina comprise ten equally numerous types (Kolb *et al*., 1981; Cohen & Sterling, 1990), so the hit rate for finding the type expressing GAD would be at best 10%. And where specific types are still sparser, $\sim$1% among diverse retinal amacrine cells (MacNeil & Masland, 1998) and cortical cells (Solnick *et al*., 1984), the problem would be worse (Fig. 1).

Ideally, one would inject only cells that demonstrably express the protein of interest. But once a cell is rendered porous to admit antibodies, it is leaky to hydrophilic dyes. We found that such cells will retain a lipophilic dye, electrophoretically deposited as a crystal, from which it then diffuses in the plasma membrane to vividly reveal cell morphology. Such dyes (DiI, DiO) have been occasionally used in this way (Lukas *et al*., 1998; Gan *et al*., 1999; Gan *et al*., 2000), but this strategy has not been broadly applied. Here we describe the technical conditions for this method and demonstrate three uses in two tissues for co-mapping specific proteins and morphology.

Materials and methods

TISSUE PREPARATION

Adult animals were deeply anesthetized: for cat and rat, 50 mg/kg pentobarbital; for guinea pig, 100 mg/kg ketamine,

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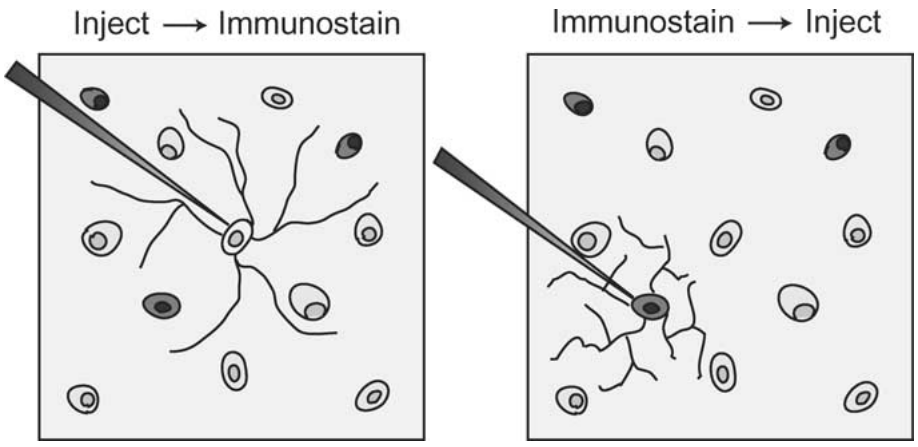

Fig. 1. The problem. *Left*: When cell injection precedes immunostaining, morphology and molecular expression rarely match. *Right*: When immunostaining precedes cell injection ("targeted injection"), this problem is solved.

20 mg/kg xylazine, and 50 mg/kg pentobarbital. Following tissue harvest, the animal was sacrificed by overdose of pentobarbital. These procedures were performed in accordance with guidelines of University of Pennsylvania and National Institutes of Health.

Retina was gently detached from the pigment epithelium and mounted on a membrane filter (type HA, Millipore, Bedford, MA). The tissue was then immersed in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 for 15 to 30 minutes. Cortical tissue was obtained from an adult rat perfused transcardially with phosphate-buffered saline followed by 4% paraformaldehyde for 30 minutes. The brain was removed and post-fixed in 4% paraformaldehyde for 1 hour at room temperature. Coronal sections of somatosensory cortex were sliced at 150 μm on a Vibratome. All tissues were stored in 0.1 M phosphate buffer pH 7.4 at 4°C.

IMMUNOCYTOCHEMISTRY

A piece of cat retina (4×4 mm^2) was incubated overnight at 4°C in 0.1 M phosphate buffer pH 7.4, containing GAD_{65} antibody (1:20, Developmental Studies Hybridoma Bank, University of Iowa), 0.3% Tween 20 as detergent (Lukas *et al.*, 1998), and 10% normal goat serum. The tissue was washed 3×10 minutes in 0.1 M phosphate buffer and then incubated for 30 minutes with 1% anti-mouse Alexa-488 (Molecular Probes) or 1% anti-mouse CY3 (Jackson ImmunoResearch), and 0.3% Tween 20. After immunostaining retina was embedded in 4% agarose, made in 0.1 M phosphate buffer pH 7.4, at 43°C, and Vibratome-sectioned at 200 μm. Rat brain slices were incubated for 30 minutes in 0.1 M phosphate buffer pH 7.4, containing parvalbumin antibody (Swant, Switzerland) at 1:2000 dilution and 0.3% Tween 20. After washing, the tissue was incubated for 30 minutes in 1% CY3 in 0.1 M phosphate buffer without detergent.

To stain all cells, the retina was immersed for 3 minutes in 0.1% solution of the nucleic acid dye, SYTO13 (Molecular Probes), in 0.1 M phosphate buffer pH 7.4.

ELECTRODES AND DYE SOLUTIONS FOR DII OR DIO

Sharp microelectrodes were pulled from borosilicate glass capillaries to ~30 MΩ (measured with 1 M KCl). The electrode tip was filled with 1% DiI in 100% ethanol, or 1% DiO in methylene chloride. The rest of the electrode was then filled with 100% ethanol. Dye was expelled by 1–50 nA positive current. The duration of dye injection varied according to cell size. A small neuron, such as the retinal bipolar cell (soma diameter ~8 μm), needed only 10 seconds at ~1 nA. A large neuron (soma diameter ~25 μm) with wide dendritic field needed up to 5 minutes at 50 nA.

Results

MORPHOLOGY OF AN IMMUNO-TARGETED RETINAL BIPOLAR NEURON

Mammalian retinas contain ten types of cone bipolar cell (Masland, 2001; Sterling, 2003). All are thought to release glutamate as their transmitter, but a few cone bipolar cells in cat retina were observed to contain GABA (Chun & Wässle, 1989) and its synthetic enzyme GAD_{65} (Vardi & Auerbach, 1995). To determine which of the ten types might be GABAergic and to trace out its circuitry, the first step would be to identify the morphology (Fig. 2).

About 10% of the cone bipolar somas stained positively for GAD_{65}, but their axonal processes, whose stratification is crucial for determining type, were lost amidst the intensely stained amacrine processes (Fig. 2). Selecting a GAD-positive bipolar soma, we electrophoresed DiI (1 nA, 10 sec). The lipophilic compound meeting the aqueous intracellular environment formed a small crystal that was trapped within the cell and from that depot diffused into the membrane. This revealed the GAD-expressing bipolar cell as a type whose axon stratifies shallowly in the inner plexiform layer (Fig. 2). Elsewhere we show that this type also expresses the vesicular GABA transporter, and glutamate plus its vesicular transporter. Thus this cell may release both transmitters (Kao *et al.*, 2004).

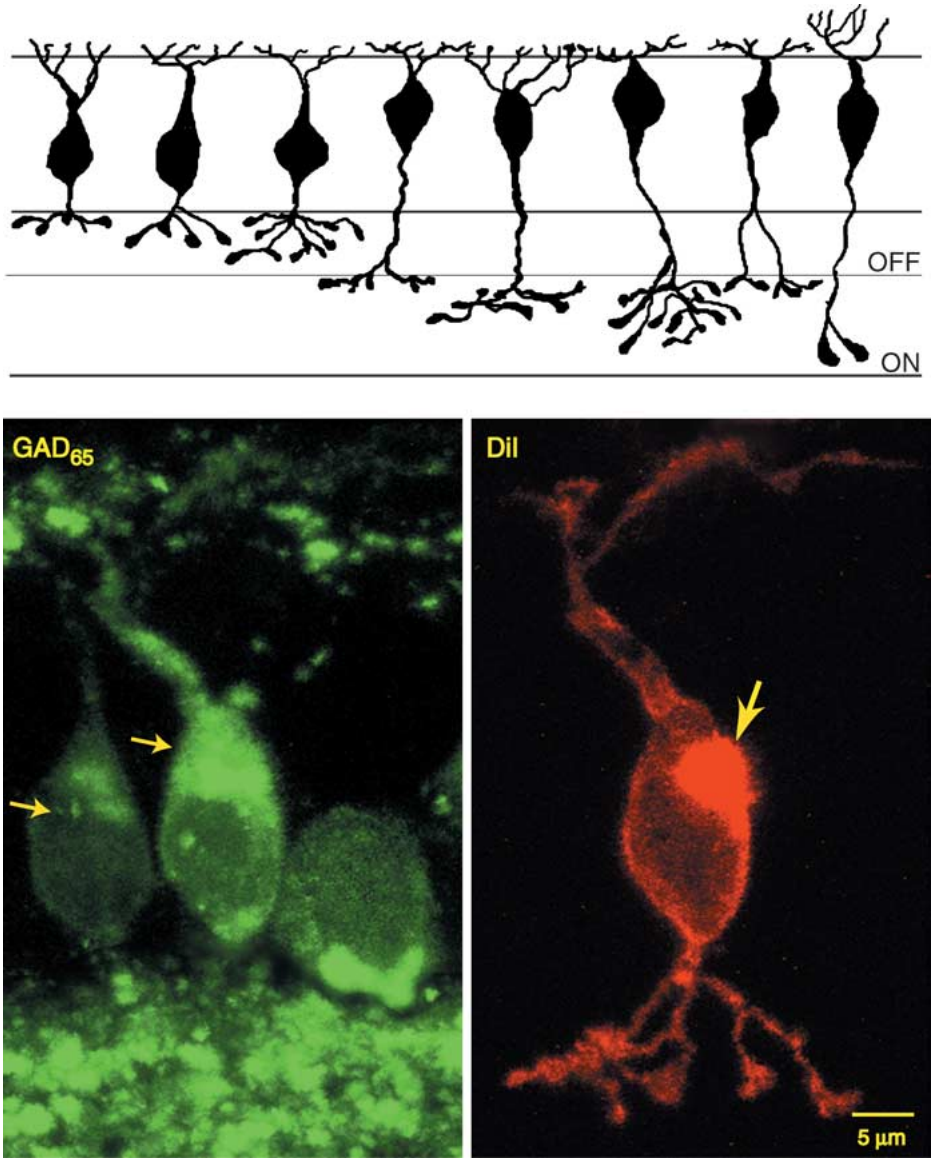

Fig. 2. Targeted injection identifies the morphology of a GABAergic bipolar cell. *Upper*: Cat retina contains many types of cone bipolar cell. *Lower left*: About 10% of the cone bipolar somas express GAD_{65}, but which types cannot be determined by immunostaining. *Lower right*: After immunostaining, GAD_{65}-positive cell was injected and identified as type OFF.

MORPHOLOGICAL DIVERSITY OF GAD_{65} AMACRINE CELLS IN THE GANGLION CELL LAYER

About half of the neurons in the ganglion cell layer of mammalian retina are actually interneurons, termed "displaced" amacrine cells, with processes confined to the inner plexiform layer (Hughes & Vaney, 1980; Vaney, 1980). The main population of amacrine cells located in the inner nuclear layer are known to be morphologically diverse (Vaney, 1990; MacNeil & Masland, 1998), but the displaced amacrine cells have not been systematically characterized. Immunostaining showed about 15% of the displaced amacrine cells to be GAD_{65}-positive (Fig. 3). Injecting these targeted neurons with lipophilic dye revealed considerable morphological diversity. For example, two neurons showed processes similar in branching pattern and extent, but the processes occupied different strata of the inner plexiform layer, an indication that their connections differ markedly (Fig. 3). A third neuron displayed extremely long, straight processes, spanning nearly a millimeter (Fig. 3). Altogether we have found that displaced amacrine cells positive for GAD_{65} comprise ten morphological types (Kao & Sterling, 2003).

CONNECTING SPECIFIC TYPES OF AMACRINE AND GANGLION CELLS

Electron microscopy shows numerous amacrine cell synapses on ganglion cell dendrites (Dowling & Boycott, 1966; Kolb, 1979; Freed & Sterling, 1988;

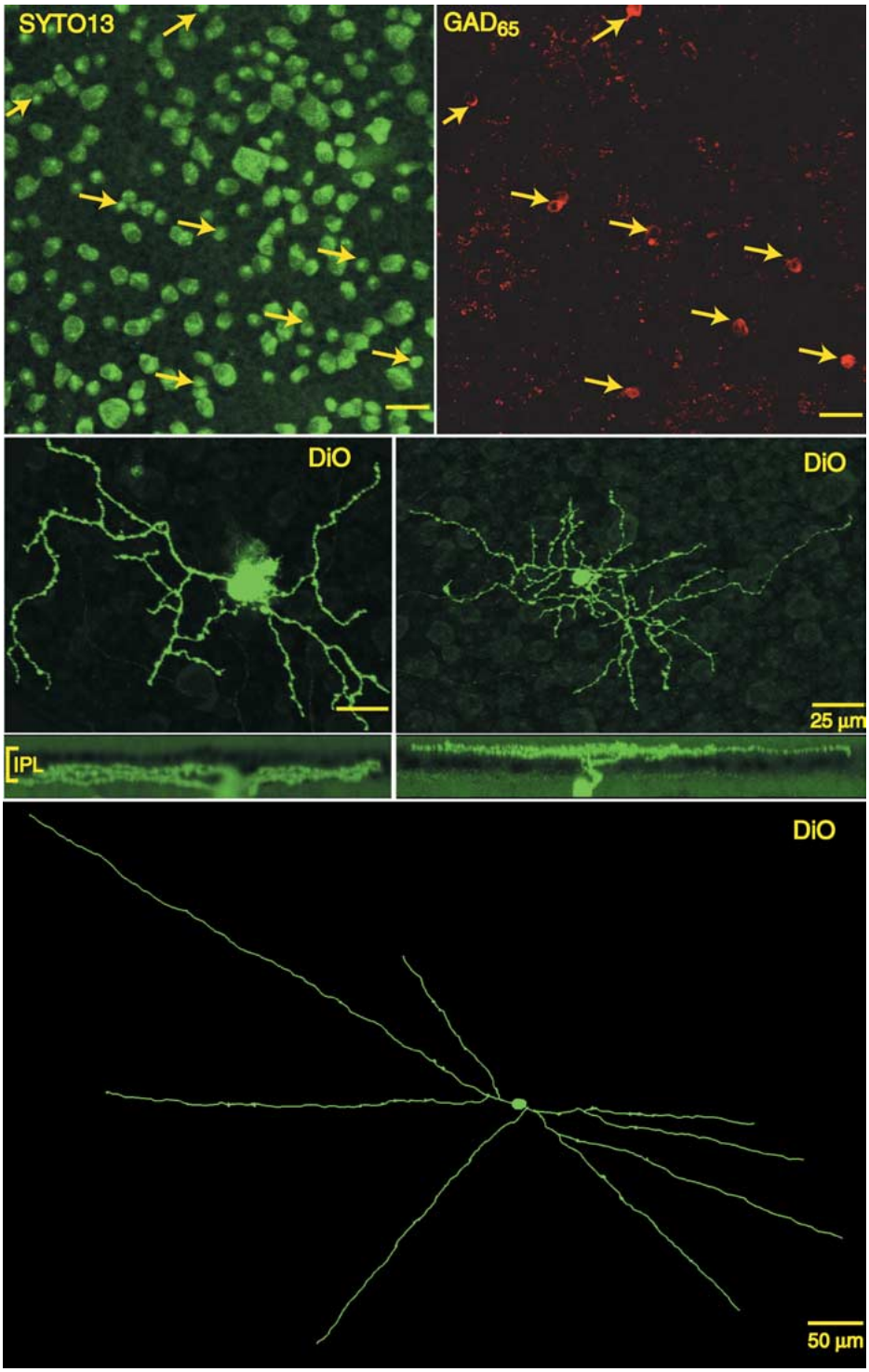

Fig. 3. Targeted injection of GAD_{65} amacrine cells reveals morphological diversity. Nucleic acid stain (SYTO13) displays all the neuron somas (180) in the ganglion cell layer. Eighty somas are amacrine, based on size ($\sim$8 μm diameter) and centrally located nucleus. Eight amacrine somas are GAD_{65}-positive, and when these are injected systematically, they prove to be morphologically diverse. Shown here are two types with similar local branching (over $\sim$250 μm) but different stratification (compare rotated views). The third cell is a long-range amacrine cell whose processes span $\sim$900 μm. Guinea pig retina, confocal images taken with 40$\times$ objective, 1.25 NA.

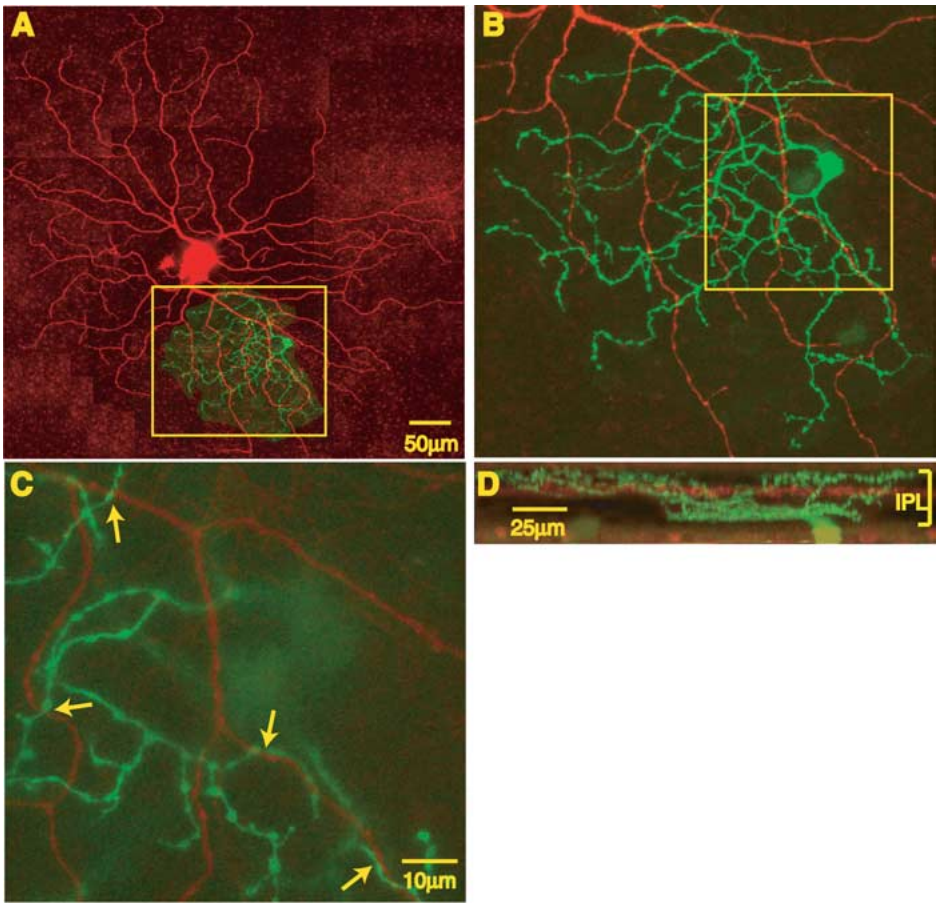

Fig. 4. Targeted injection demonstrates specific GABAergic connections to an identified ganglion cell (guinea pig). (A) Amacrine soma identified as GAD_{65}-positive was injected with DiO to reveal a narrow-field cell. The dendritic arbor of a brisk-transient ganglion cell (Demb *et al.*, 2001) was labeled with DiI. (B) Higher magnification shows intertwining of the amacrine and ganglion cell processes. 40× 1.25 NA. (C) Still higher magnification reveals probable contacts between amacrine varicosities and ganglion cell dendrites (arrows). 100× 1.4 NA. (D) Rotation (90°) of both cells. The ganglion cell dendrites stratify narrowly in the OFF layer, and the amacrine cell processes spread diffusely through the full depth of the inner plexiform layer (IPL).

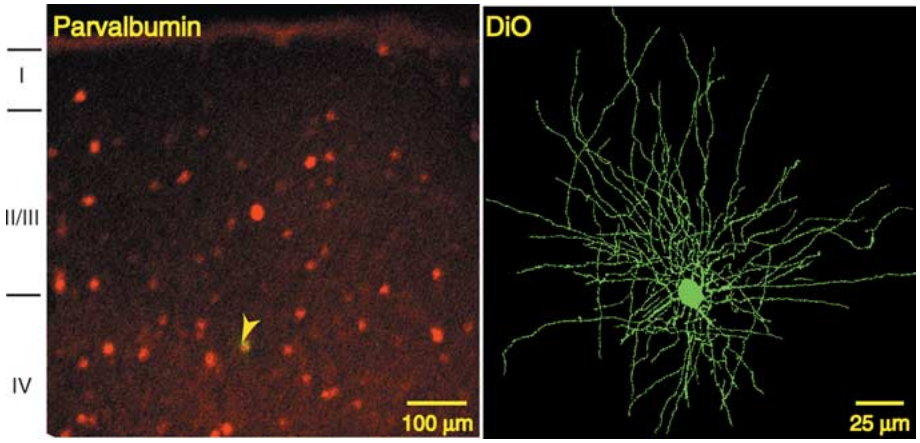

Fig. 5. Targeted injection works in a cortical slice (rat). Thirty somas in the field stained positively for parvalbumin, but this is only ∼15 % of all somas. One soma (arrowhead) in layer IV was injected with DiO, revealing a "nest basket cell".

Calkins *et al*., 1998). But which types of amacrine cell contact a particular type of ganglion cell has proved difficult to investigate. Targeted injection is well suited for this purpose and the strategy is shown in Figure 4. Retina was first immunostained to mark the population of GAD_{65} amacrine cells; then a ganglion cell was injected with DiI. After twenty-four hours when the full ganglion cell arbor was visible, we injected nearby GAD_{65} amacrine somas with DiO.

Confocal microscopy at low magnification demonstrated an amacrine cell's morphology (Fig. 4A). Higher magnification established that it intertwined with the ganglion cell dendrites over about 10% of the ganglion cell field (Fig. 4B). Still higher magnification showed numerous points of intimate contact which probably represent synapses (Fig. 4C). Finally, the image rotated into vertical view showed the ganglion cell dendrites to stratify narrowly in the OFF lamina; whereas the amacrine processes extended through the full depth of the inner plexiform layer. Thus this GABAergic amacrine type might be excited by ON bipolar cells and inhibit the OFF ganglion cell. This anatomical circuit might serve a recently identified functional circuit (Roska & Werblin, 2001; Zaghloul *et al*., 2003).

MORPHOLOGY OF RARE CELL TYPES IN THE CORTICAL SLICE

Whereas retina contains on the order of 80 cell types (Kolb *et al*., 1981; Sterling, 1983; Masland, 2001), a specific area of cerebral cortex probably contains several hundred types (Solnick *et al*., 1984; Einstein *et al*., 1987; McBain & Fisahn, 2001; Callaway, 2003). Consequently, a cell type expressing a particular protein may form an extremely small fraction of all the cells. In this case targeted injection would seem absolutely essential to identify its morphology, and we found this to be feasible.

In a slice of somatosensory cortex about 15% of all the neurons stained positively for the calcium-binding protein, parvalbumin (Fig. 5). When one small immunopositive soma in layer IV was injected with DiO, its morphology could be clearly matched to a type previously described as a "nest basket cell" (Wang *et al*., 2002). Thus, targeted injection should facilitate matching morphology to molecular expression in slices of cortex and other brain regions.

Discussion

Carbocyanine dyes (such as DiI and DiO) have frequently been used to demonstrate neuronal morphology in fixed tissue (Honig & Hume, 1989). But they have been applied only rarely following immunostaining (Lukas *et al*., 1998). Indeed, we found that rigorously permeabilizing tissue with harsh detergents, such as Triton X 100, prevents the dye from spreading in neuronal membranes. But a milder procedure, substituting a gentler detergent (Tween 20) permitted antibody penetration while preserving sufficient membrane structure to support efficient dye spread. An intracellular dye crystal 1–2 μm diameter supplies enough dye to fully label a ganglion cell dendritic arbor over 8 hours at 25°C. We have also used these dyes as a source of free radicals to "photoconvert" diaminobenzidine to an electron dense reaction product (Sandell & Masland, 1988). Thus the circuitry described here by confocal microscopy can be pursued to the EM level.

Although we have shown here four different applications of this strategy, two limitations should be acknowledged. First, some antibodies fail to penetrate under the relatively mild conditions needed for dye spread, so the method will not work for every antibody. Of course, this is also true for other methods; for example, certain antibodies that stain tissue fail on Western blots, and vice-versa. Second, these lipophilic dyes spread in *any* membrane. Consequently, the slightest contact of the electrode tip with tissue other than the targeted cell can cause diffuse contamination. This restricts the injection to intact somas at the tissue surface.

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