Dissecting and Staining *Drosophila* Optic Lobes

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**ABSTRACT**

The *Drosophila* visual system is composed of the retina and the optic lobes, which are the ganglia where photoreceptors project and initial processing of visual inputs occurs. This chapter briefly describes the different ganglia that form the visual system and provides a protocol for dissecting and staining *Drosophila* optic lobes at different stages of development (larva, pupa, and adult).

**THE DROSOPHILA VISUAL SYSTEM**

In recent years, the *Drosophila* brain has emerged as a model for studying neural circuits and the different sensory inputs that these circuits process to direct behavior (Luo et al. 2008). The *Drosophila* visual system consists of two main structures, the retina and the underlying optic lobe, that process visual information received from the photoreceptors (Tang and Guo 2001; Rister et al. 2007; Gao et al. 2008; Katsov and Clandinin 2008; Yamaguchi et al. 2008). A number of distinct optic lobe cell types have been described as a result of studies using Gal4 drivers and single neuron labeling techniques (Fischbach and Dittrich 1989; Otsuna and Ito 2006; Rister et al. 2007; Gao et al. 2008; Katsov and Clandinin 2008; Morante and Desplan 2008). In a few instances, the behavior directed by these cells has also been analyzed (Rister et al. 2007; Gao et al. 2008; Katsov and Clandinin 2008).

The enormous cell diversity observed in the visual system offers a powerful system for neurobiologists to study neurogenesis, neuronal polarity, axon guidance, and the correlation of specific cell populations with their physiological functions in driving behavior. The Protocol describes methods for the dissection and staining of *Drosophila* optic lobes that can be used to analyze the anatomy and generation of these neural circuits at any stage of development.

The *Drosophila* Retina

The *Drosophila* compound eye is composed of about 800 individual eyes called ommatidia (Wolff and Ready 1993). Each adult ommatidium contains eight photoreceptor cells (PRs) that can be classified into two groups based on their morphology and the visual input for which they specialize: two inner PRs (R7 and R8) surrounded by six outer PRs (R1–R6). R1–R6 express the wide-spectrum photopigment rhodopsin 1 (Rh1) and are important for motion detection. They can be considered homologous to the vertebrate rod cells. In contrast, R7 and R8 express different rhodopsins (Wernet and
Desplan 2004) and have a topographic organization with R7 placed on top of R8. These characteristics strongly suggest that R7 and R8 are involved in color vision, thus resembling vertebrate cone cells.

Ommatidia can be divided into three subtypes based on the rhodopsin content of their inner PRs (Wernet and Desplan 2004). The pale subtype (30%) contains ultraviolet (UV)-sensitive Rh3 in R7 and blue-sensitive Rh5 in R8. The yellow subtype (70%) contains UV-sensitive Rh4 in R7 and green-sensitive Rh6 in R8. Pale and yellow ommatidia are distributed stochastically in the main part of the retina and specialize in the discrimination of short or long wavelengths, respectively. A third subtype of ommatidia, located in the dorsal rim area, contains UV-sensitive Rh3 in both R7 and R8 and is specialized in detecting the vector of polarized light (Wernet et al. 2003).

The spatial distribution and expression of rhodopsin genes in the outer and inner PRs reflects the widely different functions of R1–R6 (motion detection) versus R7 and R8 (color vision). Indeed, the inputs transmitted by the outer and inner PRs are processed in two different targets in the optic lobes (Morante and Desplan 2004). Outer PRs project to the first optic neuropil, called the lamina, where they connect to five lamina neuron subtypes, L1–L5. Processing of information coming from R7 and R8 occurs in two distinct layers of the medulla, the second optic ganglion. R8 synapses in the superficial M3 layer, whereas R7 terminates in the deeper M6 layer. The medulla also receives inputs from L1–L5 lamina cells and thus merges motion detection and color vision.

The Optic Lobe Neuropils

The Drosophila optic lobes are formed by several structures that mediate different behaviors and represent different levels of processing: the lamina, the medulla, and the lobula complex, which is formed by the lobula and the lobula plate (Fig. 1). The optic lobes develop from two larval neuroepithelia: the inner proliferation center (IPC) and the outer proliferation center (OPC) (White and Kankel 1978) (Fig. 2A), where neuroepithelial cells increase in number through symmetric cell divisions with a plane of cell division parallel to the epithelial surface. In the “transition zones” (Egger et al. 2007; Yasugi et al. 2008), epithelial cells give rise to neuroblasts that divide asymmetrically with a plane of cell division that is now perpendicular to the epithelium to self-renew and produce differentiated optic lobe neurons (Egger et al. 2007; Yasugi et al. 2008). As a result, the IPC gives rise to the lobula complex, and distinct regions of the OPC generate the cells of the medulla cortex, medulla rim, and lamina (Meinertzhagen and Hanson 1993; J Morante and C Desplan, unpubl.) (Fig. 2).

The optic lobes represent the largest structure in the fly brain, and each lobe is formed by an estimated 60,000 neurons (Hofbauer and Campos-Ortega 1990). The optic lobes contain a very large number of cell types; for example, the medulla alone has been estimated to contain more than 60 different cell types (Cajal and Sanchez 1915; Strausfeld 1976; Fischbach and Dittrich 1989; Meinertzhagen and Sorra 2001; Otsuna and Ito 2006; Morante and Desplan 2008). How this cell diversity is generated remains mostly unknown.

FIGURE 1. Organization of the optic lobe in adult Drosophila. Optic lobe cell bodies are visualized with Elav (blue) and the neuropil is labeled with DN-cadherin (red). Photoreceptor projections in the medulla are visualized with GMR-lacZ transgene (green). (Lo) lobula; (LP) lobula plate; (Me) medulla. Lamina is missing in this picture.
Dissecting and Staining Drosophila Optic Lobes

The First Optic Neuropil: The Lamina

In the region of the OPC neuroepithelium that gives rise to the lamina, the release of Hedgehog (Hh) by photoreceptors serves as an inductive signal for proliferation of lamina precursor cells (Huang and Kunes 1996). However, Hh alone is not sufficient for assembly of the lamina cartridge. Hh induces lamina precursor cells to express the epidermal growth factor receptor (EGFR). The EGFR ligand Spitz (Spi) is transported from photoreceptors through their axons and activation of EGFR in lamina precursor cells is necessary for differentiation of the lamina cartridge (Huang and Kunes 1996; Huang et al. 1998).

The lamina is the best-characterized optic neuropil. There are an estimated 6000 cells organized in lamina cartridges (Meinertzhagen and Sorra 2001), which are the functional units in the lamina (Hofbauer and Campos-Ortega 1990). Recently, very elegant work has shown that L1 and L2 lamina neurons, which are postsynaptic targets for R1–R6, are necessary and sufficient for motion-dependent behavior (Rister et al. 2007). In contrast, L3 seems to contribute to orientation behavior but not to motion detection (Rister et al. 2007). Thus, the lamina cartridges represent the first step in visual processing as they are the direct targets for outer PRs and are involved in the initiation of the motion detection pathway.

The Second Optic Neuropil: The Medulla

The medulla, with an estimated 40,000 neurons, is the most complex optic neuropil in Drosophila (Fischbach and Dittrich 1989; Morante and Desplan 2008). Medulla cell bodies are located either in the medulla cortex (the region between the lamina and the medulla neuropil) or in the medulla rim (the region between the medulla and the lobula plate) (Fig. 1). The medulla neuropil is stratified into 10 layers (M1–M10) (Fig. 3) that can be grouped into two domains: M1–M6, where R7 and R8 send their projections, and M7–M10 in the lower medulla, which is devoid of photoreceptor projections (Fischbach and Dittrich 1989; Morante and Desplan 2008).

Photoreceptor innervation is not necessary for the development of medulla neurons. Instead, neuroepithelial cells generate neuroblasts that differentiate into the different types of medulla neurons. Expression of Lethal of scute (L[1]sc) signals the transition of OPC neuroepithelial cells to neuroblasts in a proneural wave that sweeps through the surface of the brain. This wave appears to be at least in part regulated by Janus kinase (JAK)-signal transducers and activators of transcription (STAT) signaling; JAK-STAT negatively controls the progression of this wave and thus the final number of OPC neuroblasts that will generate the medulla (Yasugi et al. 2008).
The medulla is the target for inner PRs (Takemura et al. 2008) and is thus involved in color vision (Gao et al. 2008). One prerequisite to discriminate colors is to be able to compare the inputs of at least two PRs focusing on the same point in space that are sensitive to different wavelengths of light. Drosophila contains the machinery to perform color vision, because R7 and R8 look in the same direction and contain photopigments sensitive to different wavelengths (Salcedo et al. 1999; Yamaguchi et al. 2008). Therefore, the fly color vision system likely relies on comparing the inputs of R7 and R8 in the medulla neuropil.

To understand how color vision is processed, we have described the target neurons of R7 and R8 in the medulla (Morante and Desplan 2008) in a study that builds on the pioneering work in Calliphora using Golgi impregnations (Cajal and Sanchez 1915). Using the same technique, several groups have identified many cell types in the medulla of Drosophila and Musca (Strausfeld 1976; Fischbach and Dittrich 1989). Recently, using mosaic analysis of single cells with a repressible cell marker (MARCM) (Lee and Luo 1999) and a series of enhancer-trap Gal4 lines reporting expression of several transcription factors, we have estimated that more than 60 cells participate in a single medulla column (the functional unit of the medulla) (Morante and Desplan 2008). The enormous number of cell types forming part of a medulla column contrasts with the relatively small number of elements that provide inputs into the medulla (R7 and R8 and R1–R6 through L1–L5 neurons). Thus, the divergence in the flow of information between PRs and medulla neurons argues that much local processing occurs in the medulla.

This analysis has allowed differentiation between two main classes of neurons (Morante and Desplan 2008): (1) projection neurons, both columnar and noncolumnar, that connect the medulla with the lobula only, with both the lobula and lobula plate, or with the lamina; (2) local columnar and noncolumnar interneurons, whose ramifications reside within the limits of the medulla. Although columnar medulla cells maintain the retinotopic map and receive visual inputs from only one ommatidium, noncolumnar cells are able to receive inputs from several ommatidia.

The medulla also receives inputs from the lamina L1–L5 neurons, the primary target neurons for outer PRs (Takemura et al. 2008), which suggests that it is an intermediate target in the motion detection pathway before higher processing in the lobula plate (Morante and Desplan 2008).

The Lobula Complex: Lobula and Lobula Plate

The last step in the visual pathway, at least in the optic lobe, is the lobula complex, which is formed by the lobula plate and the lobula. The lobula complex represents a high-order processing structure for visual information and has been studied in detail in large flies such as Musca domestica (Pierantoni 1976) and recently also in Drosophila (Scott et al. 2002; Otsuna and Ito 2006; Katsov and Clandinin 2008). Interestingly, lobula complex cells possess wide-field ramifications and thus may be where visual inputs received from the medulla converge.
Dissection and Staining of *Drosophila* Optic Lobes at Different Stages of Development

We outline procedures for dissecting the optic lobes from *Drosophila* larvae, pupae, and adults. We also describe methods for visualizing the anatomy of brain neural circuits by staining with fluorescent secondary antibodies and primary antibodies specific for various neuronal populations and architectural features.

### MATERIALS

**CAUTION:** See Appendix for proper handling of materials marked with <!>.  
See the end of the chapter for recipes for reagents marked with <R>.

**Reagents**

- Glycerol (50%)
- Normal goat serum (5%, v/v) <R>
- Paraformaldehyde (37%) (<Fisher; cat. no. F79500>) <!>
- PB (0.1 M phosphate buffer, pH 7.2) <R>
- PBT (0.1 M phosphate buffer containing 0.3% Triton X-100) <R>
- Primary antibodies (see Table 1)
- Secondary antibodies (see Table 2)
- Vectashield mounting medium (for preserving fluorescence) (Vector Laboratories; cat. no. H-1000)

### TABLE 1. Primary antibodies used to label *Drosophila* brains

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>To detect expression of GFP- or lacZ-based constructs:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit anti-GFP</td>
<td>Molecular Probes; A11122</td>
<td>1:1000</td>
</tr>
<tr>
<td>Sheep anti-GFP</td>
<td>Biogenesis; 4745-1051</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit anti-β-Gal</td>
<td>Cappel; 855978</td>
<td>1:20,000</td>
</tr>
<tr>
<td>Mouse anti-β-Gal</td>
<td>Promega; Z378A</td>
<td>1:500</td>
</tr>
<tr>
<td>To label relevant architectural features:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse anti-nc82 (to label general larval, pupal, and adult neuropil)</td>
<td>Developmental Studies</td>
<td>1:50</td>
</tr>
<tr>
<td>Rat anti-DE-cadherin (DCAD2) (to label the larval neuroepithelium and neuropil in larvae and early pupae)</td>
<td>Developmental Studies</td>
<td>1:50</td>
</tr>
<tr>
<td>Rat anti-DN-cadherin (DN-EX#8) (to label general neuropil in larvae, pupae, and adult)</td>
<td>Developmental Studies</td>
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<tr>
<td>To label neuronal populations in larvae:</td>
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<td></td>
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<tr>
<td>Mouse anti-24B10 (to label photoreceptors and their projections)</td>
<td>Developmental Studies</td>
<td>1:50</td>
</tr>
<tr>
<td>Mouse anti-Dachshund (mAbdac 2-3) (to label lamina precursors and lobula cells)</td>
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<tr>
<td>Mouse anti-Elav (9F8A9) (to label neurons)</td>
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</tr>
<tr>
<td>Rat anti-Elav (7E8A10) (to label photoreceptors and their projections)</td>
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TABLE 2. Secondary antibodies used to label Drosophila brains

<table>
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<tr>
<th>Secondary antibody</th>
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<tr>
<td>Donkey anti-sheep Alexa488</td>
<td>Molecular Probes; A-11015</td>
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<tr>
<td>Donkey anti-mouse Alexa488</td>
<td>Molecular Probes; A-21202</td>
<td>1:1000</td>
</tr>
<tr>
<td>Goat anti-rabbit Alexa488</td>
<td>Molecular Probes; A-21429</td>
<td>1:1000</td>
</tr>
<tr>
<td>Donkey anti-mouse Alexa555</td>
<td>Molecular Probes; A-31570</td>
<td>1:1000</td>
</tr>
<tr>
<td>Donkey anti-rabbit Alexa555</td>
<td>Molecular Probes; A-31572</td>
<td>1:1000</td>
</tr>
<tr>
<td>Goat anti-guinea pig Alexa555</td>
<td>Molecular Probes; A-21435</td>
<td>1:500</td>
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<tr>
<td>Donkey anti-rat Alexa555</td>
<td>Molecular Probes; A-21434</td>
<td>1:500</td>
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<tr>
<td>Donkey anti-mouse Alexa647</td>
<td>Molecular Probes; A-31571</td>
<td>1:500</td>
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<tr>
<td>Donkey anti-rabbit Alexa647</td>
<td>Molecular Probes; A-31573</td>
<td>1:500</td>
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<tr>
<td>Donkey anti-rat Cy5</td>
<td>Jackson Immunoresearch Laboratories; 712-175-153</td>
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<tr>
<td>Goat anti-guinea pig Alexa647</td>
<td>Molecular Probes; A-21450</td>
<td>1:500</td>
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</tbody>
</table>

Equipment

Cover glasses (18 x 18-mm and 24 x 50-mm) (Fisher Scientific; cat. no. 12-542A and 12-545F)
Dissecting microscope
Dissection dishes (three-well, glass) (Fisher Scientific; cat. no. 21-379)
Forceps (Dumont #55; Fine Science Tools)
Imaging microscope and software
Microscope slides (Fisher Scientific; cat. no. 12-550-15)
Minutien pins (0.1 mm diameter) (Fine Science Tools; cat. no. 26002-10)
Mounted pins (fine)
Nail polish (clear)
Orbital shaker (Bellco Biotechnology)
Paintbrush (small)
Pin holders (12 cm) (Fine Science Tools; cat. no. 26016-12)
Sylgard 184 Silicone Elastomer Kit (Dow Corning) (Stern 1999)

METHOD

Dissection

The dissection procedure should take ~10–25 min per experiment. It is critical to minimize dissection time to avoid tissue degradation.

1. Dissect Drosophila brains as described below according to the developmental stage desired.

Larval Brains

i. Collect larvae at the stage of interest (wandering larvae) either from the walls of the vial using forceps or with a PBS-soaked paintbrush for younger larvae. Alternatively, fill the vial with 50% glycerol and larvae will come up to the surface.

ii. Place larvae in a Petri dish layered with Sylgard transparent resin containing drops of PBS.

iii. Remove the brain lobes by holding the larval body with one pair of forceps and pulling from the larval mouth hook with a second pair. Discard the larval body. Remove the excess tissue surrounding the brain lobes using fine mounted pins.

iv. Place the clean brain lobes in a glass dissection dish well containing 180 µL of PBS. Keep the dish on ice.

A typical experiment requires 10–20 larval brains.
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Pupal Brains

i. Collect pupae at the appropriate developmental stage in a Petri dish layered with Sylgard transparent resin containing drops of PBS. Pupae can be distinguished from slow-moving third-instar larvae because they stop crawling and remain attached to the wall of the vial. White pupae are considered the earliest stage in pupal development, whereas older pupae are dark brown. To study specific pupal development stages, wait the appropriate number of hours before collecting.

ii. Submerge pupae in PBS and hold the pupal case through the abdominal part with one pair of forceps. With another pair, open the pupal case and pull the pupae out. Gently open the pupae and push the brain out. Remove excess tissue surrounding the brain lobes using fine mounted pins.

iii. Using a pair of sharp forceps held closed, place the clean brain lobes in a glass dissection dish well containing 180 µL of PBS. Keep the dish on ice. A typical experiment requires 6–10 pupal brains.

iv. Using fine pins, remove or gently separate the pupal eye to avoid interference with visualization of the pupal optic lobe. This step is critical for visualizing the medulla neuropil.

Adult Brains

i. Anesthetize adult flies of the appropriate genotype. Decapitate and place the heads in a glass dissection dish well containing PBS. Keep the dish on ice.

ii. Perform the dissection in a Petri dish layered with Sylgard transparent resin containing drops of PBS, or alternatively, in the dissection dish well containing PBS.

iii. Holding the head through the maxillary cavity, submerge it in PBS, and remove the maxillary palps with forceps. Hold both sides of the maxillary cavity and gently pull the two pairs of forceps away from each other to open the head cuticle. Remove the brain from the head cuticle and remove any excess surrounding tissue using fine mounted pins.

iv. Using a pair of sharp forceps held closed, place the clean adult brain lobes in a glass dissection dish well containing 180 µL of PBS. Keep the dish on ice. A typical experiment requires 6–10 adult brains.

v. Remove the retina and lamina to avoid interference with the visualization of the medulla/lobula complex and poor antibody penetration. This step is critical.

Fixation

The fixation and staining of brains should take ~1.5 d.

2. Add 20 µL of 37% paraformaldehyde to the glass wells containing 180 µL of PBS and the dissected tissue. Fix the larval, pupal, or adult brains by incubating on an orbital shaker with gentle agitation for 20 min at room temperature.

3. Remove the paraformaldehyde and wash with agitation three times in fresh PBS for 5 min each.

Staining

Day 1

4. Add 200 µL of blocking solution (5% normal goat serum) and incubate the fixed brains on an orbital shaker with gentle agitation for at least 30 min at room temperature in darkness.
5. Dilute primary antibodies in PBT in 1.5-mL tubes (you will need 200 µL per well).

6. Remove the blocking solution and add primary antibody. Incubate with agitation overnight at room temperature in darkness.

   For the majority of commercial antibodies, it is possible to omit the blocking step and incubate the dissected brains directly in primary antibodies overnight.

Day 2

7. Remove primary antibody and wash three times in PBS for 20 min each at room temperature.

   Primary antibodies can be reused three times or more.

8. Dilute secondary antibodies in PBT in 1.5-mL tubes (you will need 200 µL per well). Add secondary antibodies to brain preparations. Incubate on an orbital shaker with agitation for 3 h at room temperature in darkness.

   Prepare the secondary antibodies fresh and discard after use.

9. Remove secondary antibodies and wash three times in PBS for 20 min each at room temperature.

Mounting

Use the bridge method illustrated in Figure 4 to mount Drosophila brains and preserve their three-dimensional configuration. This procedure should take ~5–10 min per experiment.

10. Add two 2-µL drops of 50% glycerol to each end of a microscope slide. Cover these drops with 18 x 18-mm cover glasses.

11. Add a 5-µL drop of Vectashield mounting medium in between the two cover glasses.

12. Using a pair of sharp forceps held closed, place the brains in the mounting medium. Using fine mounted pins, align all the brains in the same orientation for ease of imaging.

13. Place a 24 x 50-mm cover glass on top of the bridge to cover the brains.

14. Seal the edges of the cover glass with nail polish and store the samples at 4°C in a dark slide holder.

![Figure 4](image_url)

**FIGURE 4.** Bridge method for mounting Drosophila optic lobes.
Dissecting and Staining Drosophila Optic Lobes

RECIPIES

CAUTION: See Appendix for proper handling of materials marked with <!>. Recipes for reagents marked with <R> are included in this list.

Normal Goat Serum (5%, v/v)

Add 50 µL of normal goat serum (Lampire Biological Laboratories; cat. no. S2-0609) to 950 µL of PBT <R>. Aliquot the serum in very small volumes and store at −20°C.

PB (0.1 M Phosphate Buffer, pH 7.2)

Prepare two stock solutions:

i. Sodium phosphate dibasic (0.5 M). Dissolve 35.5 g of sodium phosphate dibasic in a final volume of 500 mL of distilled H₂O. Some crystallization will occur when the solution is stored at 4°C. Warm on a hot plate and stir until the crystals dissolve.

ii. Sodium phosphate monobasic (0.5 M). Dissolve 30 g of anhydrous sodium phosphate monobasic in a final volume of 500 mL of distilled H₂O.

Prepare in separate beakers:

i. 80 mL of 0.5 M sodium phosphate dibasic stock solution and distilled H₂O to give a final volume of 400 mL.

ii. 30 mL of 0.5 M sodium phosphate monobasic stock solution and distilled H₂O to give a final volume of 150 mL.

Bring the above dibasic solution to pH 7.2 using the monobasic solution.

PBT (0.1 M Phosphate Buffer, pH 7.2, Containing 0.3% Triton X-100)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 500 mL)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB (0.1 M, pH 7.2) &lt;R&gt;</td>
<td>498.5 mL</td>
<td>1x</td>
</tr>
<tr>
<td>Triton X-100 (Sigma-Aldrich; cat. no. 9002-93-1) &lt;!&gt;</td>
<td>1.5 mL</td>
<td>0.3% (v/v)</td>
</tr>
</tbody>
</table>

Store at room temperature.

REFERENCES


