Stripe assay to examine axonal guidance and cell migration

Bernd Knöll[1], Christine Weinl[1], Alfred Nordheim[1] & Friedrich Bonhoeffer[2]

[1]Interfaculty Institute for Cell Biology, Department of Molecular Biology, University of Tübingen, Auf der Morgenstelle 15, 72076 Tübingen, Germany. [2]Max-Planck-Institute for Developmental Biology, Spemannstrasse 35, 72076 Tübingen, Germany. Correspondence should be addressed to B.K. (bernd.knoell@uni-tuebingen.de).

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Stripe assays have been widely employed as *in vitro* test systems to study the responses of growing axons, as well as migrating cells, to established or novel guidance molecules. We provide detailed protocols for both the original and the modified version of this assay, as they allow the analysis of the 'guidance properties' of active components present in crude membrane fractions or as purified molecules. Silicon matrices are used to produce striped patterns of active molecules on a surface (referred to as 'carpet'), followed by culturing of neurons, or any other cell type, on these carpets. After 1–2 days in culture, striped outgrowth of extending neurites—indicative of guided migration of cell processes—can be observed. We also discuss potential other applications (e.g., in neuronal regeneration and development) and modifications of the assay. The preparation of 10–12 carpets takes approximately 4–5 h.

INTRODUCTION

The stripe assay was originally designed by F. Bonhoeffer and co-workers[1,2] in the late 1980s to analyze fundamental axonal guidance mechanisms governing the formation of the topographic map established in the chick retino-tectal system (for review, see ref. 3). Employing this assay, the presence of a graded distribution of repulsive axon guidance cues, present predominantly in posterior tectal membranes, was discovered. Subsequently, this assay was modified, permitting the identification of ephrin-As as the key components present in posterior membranes that are responsible for the repulsive properties[4–7].

The stripe assay has since been used to uncover guidance cues and mechanisms in many principal neuronal projections, including, for example, the hippocampal, thalamic and olfactory systems[8–11]. The stripe assay was also applied to investigate neurite outgrowth and guidance behavior related to neuronal regeneration[12–15] and branch formation[16]. Furthermore, the stripe assay has proven valuable in dissecting signaling cascades initiated downstream of axonal guidance receptors activated by ligands printed in a striped pattern[12–15]. In addition, the guidance properties of membrane-tethered molecules as well as the short-range action of secreted guidance molecules (such as Slits and Semaphorins) could be mimicked *in vitro* by this assay[17,18]. Also stripe assay experiments were conducted to unravel potential guidance properties of extracellular matrix components[19,20] and of cell adhesion molecules of the IgG superfamily[21].

Of note, the stripe assay was employed as an *in vitro* assay to investigate the navigation responses of both axonal growth cones toward guidance cues and of migrating cells[4,22,23]. With respect to the latter, neural crest cells[22] and oligodendrocytes[23,24], but also non-neuronal cells such as tumor cells, have been studied[25].

The assay allows assessment of guidance activity of established molecules and also testing potential guidance properties of hitherto uncharacterized molecules such as RGM, the Wnt signaling inhibitor SFRP1 or the morphogen Sonic hedgehog[26–28].

Besides the widely used stripe assay consisting of straight individual stripes, other geometric patterns—such as zig-zag patterns—have been employed[29]. These zig-zag stripes are valuable when analyzing cell behavior and signal-transduction events at substrate borders, where, for example, one part of a cell is adhering to a laminin substrate and the other part is contacting a substrate of different molecular nature[30]. Also, a novel technique allowing for microcontact printing of proteins on surfaces has been reported and offers a versatile tool to study the behavior of, for example, navigating growth cones in gradients of axon guidance cues[31].

In the experimental setup, striped carpets are produced with the aid of specially manufactured silicon matrices to glass, plastic or nucleopore membrane surfaces.

These matrices consist of a channel system, which can be filled with a solution containing the guidance cue under investigation.

The original version of the assay employed stripes generated with crude membrane fractions of tissue (e.g., a certain brain area) or of cells transfected with the guidance molecule of interest. These crude membrane fractions are then sucked in a striped pattern onto polycarbonate nucleopore filters by application of vacuum.

The modified stripe assay allows one to examine the 'guidance properties' of purified molecules. Here, matrices are attached to either glass coverslips or plastic Petri dishes, which provide a solid and even surface to prepare the striped pattern. The choice of using either a coverslip or a Petri dish is at the experimenter's convenience. A detailed protocol is given for plastic Petri dishes, but glass coverslips have been used regularly and successfully by other investigators (see, e.g., ref. 18). After attaching the matrix to the Petri dish, the matrix channels are filled with the molecules of interest and thereby establish the set of first stripes (see **Figs. 1–3**). After incubation, the matrix is removed and the gaps between the first stripes are filled with a solution containing control molecules, thereby generating the set of second stripes. This experimental regimen results in alternating stripes consisting of guidance cue ("first stripes") and control molecule ("second stripes"). To distinguish the set of first stripes from the neighboring second stripes, the first stripe solution contains fluorescently labeled antibodies or beads. To provide a general growth-promoting environment, laminin (or any other extracellular matrix component) is homogeneously applied to the carpets. After adding, for example, neurons on top of these carpets and allowing 1–2 days of culture

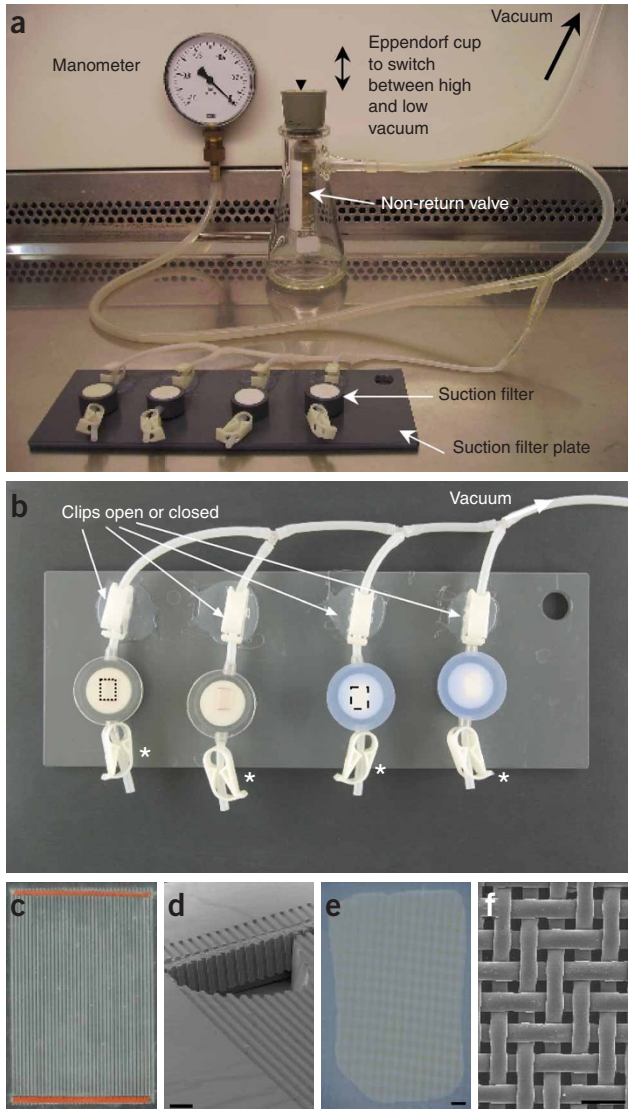


Figure 1 | Original version of the stripe assay. (**a**) Experimental setup to produce alternating stripes of membrane fragments. Four suction filters mounted on a suction filter plate are connected to a vacuum pump via a non-return valve. Closing the hole (at black triangle) of the valve by inserting an Eppendorf tube leads to an increase of the pressure in the channel system (−800 to −1,000 mbar; 'high vacuum'). Removal of this tube decreases the pressure (−200 to −400 mbar; 'low vacuum'). (**b**) Detailed view of the suction filter plate illustrating the mounting of the matrices on the suction filters. Two filters are covered with white matrices (left side) to produce the set of first stripes. The other two filters, covered with blue matrices (right side), are used for generating the set of second stripes. Clips marked with white stars should always be closed when vacuum is to be generated. Clips on top are either closed (during changing nucleopore filters) or open (during generation of the striped pattern by applying vacuum or during washing steps). The dotted and dashed rectangles on the white and the blue matrix, respectively, indicate the position taken for the higher magnifications depicted in (**c**) and (**e**). (**c**) Higher magnification of a white matrix showing the channel system. Scale bar, 100 µm. (**d**) Electron microscopic picture of the white matrix channel system at even higher magnification. Scale bar, 350 µm. (**e**) Higher magnification of the blue matrix used to prepare the second stripes, depicting the innermost white mesh and the surrounding blue silicon base. Scale bar, 100 µm. (**f**) Even higher magnification of (**e**) highlighting the mesh-like structure of the innermost part of the blue matrix. Scale bar, 100 µm.

time for neurite outgrowth/cell migration, stripe assay results can be evaluated. Preferential growth on the set of first (fluorescent) stripes demonstrates the presence of an attractive guidance cue on these stripes, whereas growth on the second stripes (containing the control molecule) is indicative of a repulsive activity of the guidance molecule placed on the first stripes. On top of that it should be kept in mind that a 'repulsive activity' as observed in the stripe assay could be due to a general non-permissive substrate property of the molecule under investigation. To demonstrate an active repulsive turning away of an individual growth cone from a local source of the guidance cue, the turning assay is widely employed within the axon guidance field (see ref. 32).

Herein we give a detailed outline of the stripe assay procedures using the chick retino-tectal projection. This protocol was adapted by various research groups for their experimental designs and, for these modifications, we would like to refer throughout the manuscript to the relevant primary research literature[5,6,9,10,14,18,33]. First we describe the guidance behavior of axons from chick retinal ganglion cells (RGCs) growing on carpets consisting of alternating membrane fractions derived from anterior and posterior chick optic tecta (original version, Step 1A of PROCEDURE). Second, a protocol describing the modified version of the stripe assay is provided (Step 1B of PROCEDURE), whereby alternating stripes of ephrinA5-Fc fusion proteins ('first stripes') and control stripes consisting of Fc alone ('second stripes') are prepared. Ephrin-As proteins trigger predominantly growth cone repulsion by activating EphA receptor tyrosine kinases on retinal axons[34,35]. Therefore, RGC axons are repelled by the set of first stripes containing ephrin-A5, leading to axonal growth congruent with the control set of second stripes.

MATERIALS

REAGENTS

- Animals/embryos: E6 and E9 white Leghorn chick embryos for preparation of RGC cultures and tectal membranes, respectively **! CAUTION** All animal experiments must comply with national regulations.
- Anti-human IgG, Fc-specific (Sigma, cat. no. I2136)
- Anti-human IgG, Fc-specific Cy3-conjugated (Sigma, cat. no. C2571)
- Aprotinin (Sigma, cat. no. A1153)
- EphrinA5-Fc, human (R&D systems, cat. no. 374-EA)
- Fc (Calbiochem, cat. no. 401104)
- Fluorescent microspheres, 0.5 µm, for example red fluorescent (Molecular Probes, cat. no. F-8812)
- Hank's balanced salt solution (HBSS; without Ca^{2+} and Mg^{2+} (Invitrogen; cat. no. 14170-088)
- Laminin (mouse, Invitrogen, cat. no. 23017015)
- Leupeptin (Sigma, cat. no. L2884)
- Methanol
- PBS sterile (Invitrogen; cat. no. 14190), add 1× penicillin/streptomycin
- Pepstatin (Sigma, cat. no. P4265)
- Spermidine (Sigma, cat. no. S2501)
- SDS, 2% solution in H_2O
- Sucrose

EQUIPMENT

- 60 mm bacterial Petri dishes (Greiner, cat. no. 628102)
- Centrifuge, suitable for Falcon tube placement
- Cell scraper
- Falcon tubes, 15 and 50 ml

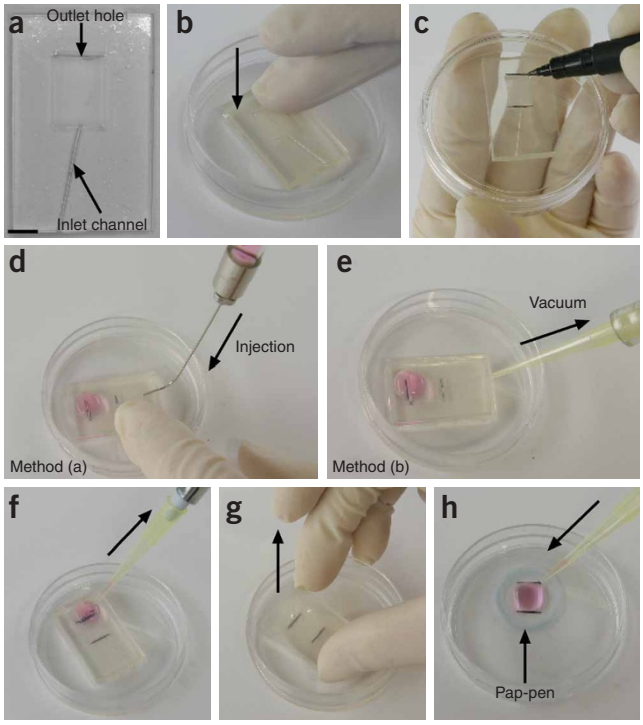
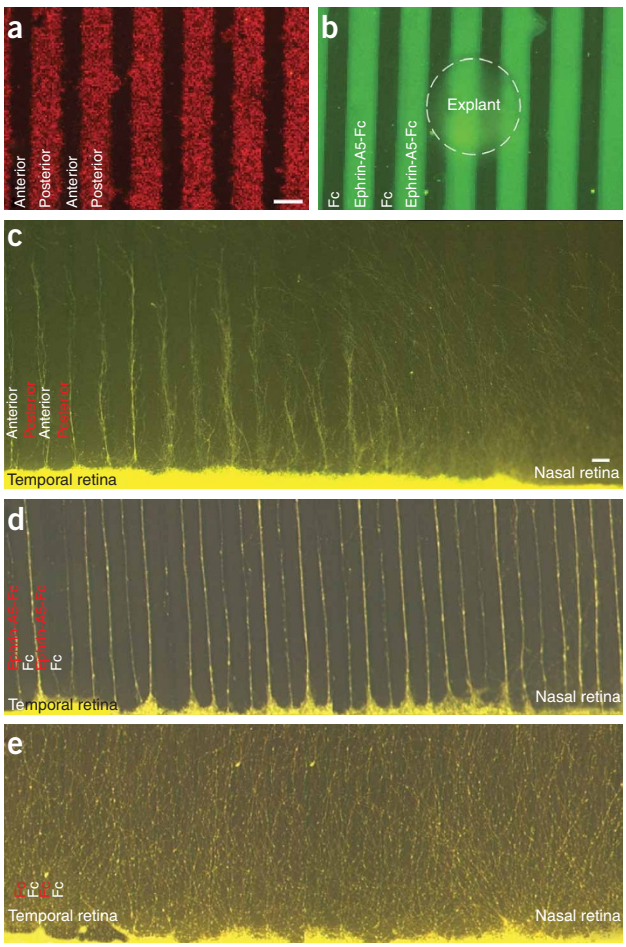


Figure 2 | Modified version of the stripe assay. (**a**) Silicon matrix used to produce stripe patterns using purified proteins. Arrows point toward the inlet channel and the outlet hole. Scale bar, 0.5 cm. (**b**) Silicon matrices shown in (**a**) are pressed firmly with channels facing downwards (arrow) onto the surface of a Petri dish. (**c**) Margins of the stripes are marked on the bottom of the dish. (**d**) Illustration of method (a) to generate stripe patterns by injecting solution into the inlet channel with the help of a Hamilton syringe. The injected solution slowly fills and passes the channels and the excess volume exits at the outlet hole. During injection, the matrix is constantly pressed onto the dish surface. (**e**) Alternative method (b) of producing striped patterns by the use of suction. A volume of 100 µl of the first solution is pipetted onto the so-called 'outlet hole' and vacuum is applied at the 'inlet' channel. (**f**) After 30 min of incubation at 37 °C, excess solution is removed by washing with HBSS (method a or b). Excess liquid at the outlet hole is removed. (**g**) Starting at the upper right corner, the matrix should be lifted off the Petri dish and removed quickly. (**h**) A volume of 100 µl of second stripe solution is applied to the delineated area. Special care has to be taken not to touch or scrape the carpet surface with the pipette tip. Therefore, the solution is applied from one corner of the marked area. Subsequent washing steps and application of laminin or culture medium are performed in a similar way. The stripe pattern is encircled with a Pap-pen to reduce the volume of culture medium required.

- Hamilton syringe (Hamilton)
- Laminar flow hood
- Needle 26G, 0.45 µm
- Silicon matrices (see EQUIPMENT SETUP)
- Pap-pen (Invitrogen, Abcam) ▲ **CRITICAL** Test Pap-pen for cytotoxicity before use.
- Polycarbonate Nucleopore membranes, 0.1 µm (Whatman, cat. no. 112105). Cut nucleopore membrane into rectangles (approximately 1.5–1.7 cm × 1.2–1.4 cm). Label rectangles by cutting the upper left corner with shiny side facing upwards
- Rotor tubes, 1.5 ml (Beckman)
- Spectrophotometer (Pharmacia Ultraspec3000), 1 ml quartz curvette
- Syringes, 1 ml
- Ultracentrifuge (TL-100 Beckmann with TLS-55 rotor)
- Equipment for suction apparatus: vaccum source (e.g., vacuum pump, laboratory bench vacuum); bottle; non-return valve fitting in bottle; tubing to connect bottle, plastic clips to close tubing (e.g., VWR), vacuum pump, suction filter plate and manometer



REAGENT SETUP

Homogenization buffer (HB) 10 mM Tris/HCl, pH 7.4, 1 mM spermidine × 3 HCl and 1.5 mM $CaCl_2 \cdot 2H_2O$. Store at 4 °C.

Aprotinin Dissolve 25 mg in 4.2 ml autoclaved H_2O, store aliquots at −20 °C; final concentration 200 U ml^{-1}.

Leupeptin Dissolve 25 mg in 26.3 ml autoclaved H_2O, store aliquots at −20 °C; final concentration 50 mM.

Pepstatin Dissolve 25 mg in 18 ml methanol, store aliquots at −20 °C; final concentration 2 mM.

50% (w/w) sucrose solution Dissolve 50 g of sucrose in 50 g homogenization buffer, sterile-filter (0.4 µm) and store at −20 °C.

5% (v/v) sucrose solution Dilute 50% sucrose solution 1:10 in homogenization buffer.

EQUIPMENT SETUP

Silicon matrices Silicon matrices and additional equipment for the stripe assay are presently distributed exclusively by the laboratory of Dr. Martin Bastmeyer

Figure 3 | Typical stripe patterns obtained. (**a**,**b**) Typical patterns of stripes obtained with the original method (**a**) (red stripes) and with the modified method (**b**) (green stripes). The circle in (**b**) delineates the position of an explant on top of this carpet. Scale bar, 100 µm. (**c**) Result of an original stripe assay experiment. Temporal RGC axons are repelled by posterior membranes (red) leading to growth on stripes derived from the anterior optic tectum. In contrast, nasal RGC axons are not repelled by posterior membranes thus growing randomly. Scale bar (**c**–**e**), 100 µm. (**d**) Result of a modified stripe assay experiment. Alternating stripes of ephrinA5-Fc (red) and Fc alone were prepared. Both temporal and nasal RGC axon populations are repelled, thus growing on the non-labeled dark set of second stripes. (**e**) Result of a control experiment of the modified stripe assay. All stripes consist of Fc alone, resulting in random RGC axon outgrowth.

(Zoologisches Institut, Universität Karlsruhe, Germany) (bastmeyer@bio.uka.de). For the original version of the stripe assay outlined in Step 1A of PROCEDURE, four white matrices to prepare the set of first stripes, four blue matrices to prepare the set of second stripes and the suction filter plate are required (see also **Fig. 1**). For the modified version of the stripe assay, outlined in Step 1B of PROCEDURE, the number of matrices (see **Fig. 2a**) required equals the number of carpets to be analyzed in one experiment. Regularly, we prepare up to 12 carpets in one experiment.

PROCEDURE

1| In the following steps we describe the procedure for the original version of the stripe assay in (A) and the modified version of the stripe assay in (B). For the stripe assay using purified proteins rather than crude membrane fractions[1] (B), a modified protocol of the initially published work is employed[4,36]. In option B we describe a protocol preparing carpets of purified proteins belonging to the ephrin-A family of repulsive axon guidance molecules[34,35]. Ephrin-As are commercially available as fusion proteins (see MATERIALS section), containing the Fc part of the human IgG. Oligomerizing (so-called 'Clustering') of Ephrin-A-Fc with anti-Fc-specific IgG enhances the repulsion towards cells and growth cones by ephrin-A5. In principle, there are two options to produce stripes using the modified version of the stripe assay: injection of first stripe solution with a Hamilton syringe into the inlet channel (**Fig. 2d**) or by applying vacuum at the 'inlet channel' to suck solution through the channel system (**Fig. 2e**).

▲ CRITICAL STEP All steps, if not indicated otherwise, are performed in a laminar flow hood.

(A) Stripe assays using crude membrane fractions (original version)

(i) **Preparations before starting.** Boil (for 5 min) and store blue and white matrices in autoclaved H_2O.

(ii) Boil (for 5 min) and store precut nucleopore filters in autoclaved H_2O.

(iii) Coat the boiled nucleopore filters with 20 μg ml^{-1} laminin (in HBSS) and incubate for at least 1 h at 37 °C. Before use, wash with 1× HBSS and store in PBS.

▲ CRITICAL STEP Thaw laminin slowly on ice (approximately 1 h) to prevent formation of aggregates.

(iv) Prepare PBS with protease inhibitors (hereafter called PBS+) by adding 250 μl leupeptin, 100 μl aprotinin and 10 μl pepstatin to 10 ml PBS; store at 4 °C (prepare freshly for each experiment).

(v) Prepare homogenization buffer with protease inhibitors (hereafter called HB+) by adding 125 μl leupeptin, 50 μl aprotinin and 5 μl pepstatin to 5 ml homogenization buffer; store at 4 °C (prepare freshly for each experiment).

(vi) Thaw 50% sucrose solution, prepare 5% sucrose solution and store on ice.

(vii) Sterilize 1.5 ml rotor tubes under UV light for 10 min.

(viii) **Preparation of crude membrane fractions.** Prepare crude membrane fractions as a source of guidance molecules. As an example, in the following steps, we describe the preparation of membrane fractions from the anterior and the posterior thirds of six optic tecta of three E9 chick embryos. This amount of tissue represents approximately a total of 40–60 mg of cell material. The optimal amount of starting material has to be determined according to the individual experimental design. Alternatively, membrane fractions harvested of tissue culture cells transfected with a guidance cue of interest can be used (see ref. 5).

(ix) Wipe the surface of chick egg with 70% ethanol and open the chick egg with blunt forceps.

(x) Transfer the embryo into a Petri dish with HBSS.

(xi) Remove the pia mater and dissect the two underlying tecta.

(xii) Cut each tectum in three parts, discard the middle part and collect the anterior and the posterior thirds of both tecta in HB+ separately.

(xiii) Wash the tectal thirds with 500 μl cold HB+.

(xiv) Add 600 μl fresh HB+ and homogenize tissue several times using a 1 ml syringe with a 26G needle.

▲ CRITICAL STEP Avoid formation of air bubbles during homogenization. Homogenize slowly, that is, allow approximately 5–10 s for 600 μl homogenate to pass through syringe.

(xv) **Sucrose gradient centrifugation.** This and the following steps describe sucrose gradient centrifugation, which will remove nuclei and cytoplasmic proteins and retain, for example, cytoplasmic and mitochondrial membrane fragments in the supernatant. First pipet 350 μl of 50% sucrose solution into a 1.5 ml rotor tube.

(xvi) Layer slowly 150 μl of 5% sucrose solution on top.

(xvii) Load a maximum of 800 μl homogenized membranes on top of the 5% sucrose cushion. The maximal amount of homogenate that can be separated represents the anterior/posterior thirds of three E9 embryos. When using transfected cells, for example HEK293 cells, the homogenate of four 10 cm dishes can be loaded[5].

(xviii) Centrifuge at 52,000*g* for 10 min at 4 °C using the TLS-55 rotor in a Beckmann TL-100 centrifuge. Meanwhile prepare Eppendorf tubes with 1 ml PBS+ and store on ice.

(xix) After centrifugation, carefully remove the tubes from the rotor. Verify—by visual inspection—the presence of a white layer at an intermediate position in the rotor tube, which contains the crude membrane extract.

(xx) Carefully transfer the white intermediate phase into the tube with 1 ml PBS+.

(xxi) Centrifuge again to remove the remaining sucrose for 7 min at 13,000*g* at 4 °C.

(xxii) Discard the supernatant and resuspend the pellet in 1 ml cold PBS+ with the syringe and 26G needle.

(xxiii) **Measurement of membrane fraction concentration.** Add 20 µl of each sample to 980 µl of 2% (w/v) SDS solution (prepare duplicates). Pipette 20 µl PBS+ to 980 µl 2% SDS as reference. Determine OD at 220 nm in a spectrophotometer using a quartz curette.

(xxiv) Multiply average OD_{220nm} value by 3.33 to get the final OD_{220nm} . 1 OD_{220nm} = 700 µg ml^{-1}. The average OD_{220nm} value of three E9 tecta resuspended in 1 ml PBS+ (see above) is approximately 0.2.

■ **PAUSE POINT** Aliquots of membrane fractions can be stored at –80 °C or in liquid nitrogen for several weeks, up to 1 month. Upon thawing, the activity should be measured again and those membranes should not be frozen again. We recommend to continue without delay, as membrane fractions lose some activity upon storage.

(xxv) Dilute membrane fractions with cold PBS+ to an OD_{220nm} of 0.1 and store on ice. Prepare 150 µl of membrane solution for preparing one set of first stripes and 300 µl of solution for preparation of one set of second stripes. As an estimate, adjusted membrane fractions with an OD_{220nm} of 0.1 contain approximately 60–70 µg protein per ml as determined by Bradford assay.

(xxvi) Label the first stripe solution with fluorescent beads by diluting 10 µl of beads in 500 µl PBS+. For 1 ml of membrane solution, add 50 µl of the diluted beads.

(xxvii) **Preparation of carpets.** Set up the vacuum-sucking device according to **Figure 1a**. Use sterile blunt forceps to handle matrices and nucleopore membranes.

(xxviii) **Preparation before getting started with stripe preparation.** Sterilize the suction filter plate by spraying with 70% EtOH; rinse individual suction filters with PBS (see **Fig. 1b**).

(xxix) Check the vacuum with all four clips closed: at 'low' vacuum: −200 to −400 mbar (no Eppendorf tube in the bottle; see **Fig. 1a**); and at 'high' vacuum: −800 to −1,000 mbar (insert Eppendorf tube in the bottle). Closing or opening a hole in the bottle lid with the Eppendorf cup allows for switching between low and high vacuum.

(xxx) Prepare 10 cm dishes with individual PBS+ drops to store each nucleopore membrane on top after the first stripes have been prepared. The PBS+ drop underneath the membrane will prevent the membranes from drying out.

(xxxi) Prepare 35 mm Petri dishes with one drop of PBS+ in the middle to store carpets after preparation of second stripes.

(xxxii) **Preparation of first stripes.** Cover each suction filter with one white matrix and rinse all matrices with 1 ml of PBS. Red lines of white matrix should be facing upward (see **Fig. 1b,c**).

(xxxiii) Close all clips.

(xxxiv) Add one nucleopore membrane on each of the four suction filters, with the marked corner of the membrane oriented to the upper left side (shiny surface faces downwards).

(xxxv) Pipette 150 µl of first stripe solution on each of the four nucleopore membranes.

(xxxvi) Open clips, and apply high vacuum by closing the hole of the vacuum bottle with an Eppendorf tube.

(xxxvii) Start timer at −600 mbar and allow to suck for 90 s.

(xxxviii) Remove excess liquid and stop high vacuum by removing the Eppendorf tube.

(xxxix) Leave low vacuum until stripes are visible on filter surface.

(xl) Immediately close clip and transfer all four nucleopore membranes on PBS+ drops in the Petri dish.

(xli) The quality of the first set of stripes can be quickly checked by fluorescence microscopy.

(xlii) Wash the white matrices before mounting the next nucleopore membranes by opening clips and rinsing with 1 ml of PBS.

▲ **CRITICAL STEP** Do not allow nucleopore membranes to dry out at any time.

(xliii) **Preparation of second stripes.** Wash the suction filters three times with 1 ml of PBS by applying vacuum.

(xliv) Add one blue matrix on each suction filter (in either orientation).

(xlv) Lift the blue matrix slightly at the edge and suck with 1 ml PBS+ underneath.

▲ **CRITICAL STEP** Make sure blue matrices are lying completely flat on suction filters (no air bubbles underneath).

(xlvi) Transfer nucleopore membranes (stored on PBS+ drop) onto suction filters.

(xlvii) Immediately add 150 µl of second stripe solution and open clip; leave low vacuum until all four clips are opened.

(xlviii) Apply high vacuum (insert Eppendorf tube) and suck for 90 s once −600 mbar is reached.

▲ **CRITICAL STEP** Do not allow nucleopore membranes to dry out at any time.

(xlix) Rinse nucleopore membranes with 1 ml of PBS+.

(l) Close clip and stop the high vacuum (remove Eppendorf tube).

(li) Transfer processed nucleopore membranes to individual 35 mm Petri dishes (with a drop of PBS+ at the bottom).

(lii) Add one drop of tissue culture medium onto the nucleopore membrane.

■ **PAUSE POINT** Store dishes at 37 °C and use on the same day (i.e., as soon as cells are ready to be cultured on carpets).

(B) Stripe assays (modified version) using purified proteins

(i) **Preparations before starting experiment.** Remove dust from matrix stripe surface with transparent tape. Boil matrices for 5–10 min in autoclaved H_2O and dry overnight with striped pattern facing upwards in a laminar flow hood.

▲ **CRITICAL STEP** Matrices should be completely dry before being used.

(ii) Sterilize 60 mm bacterial dishes under UV for 5–10 min.

(iii) Thaw laminin aliquot slowly on ice.

▲ CRITICAL STEP This step has to be performed slowly on ice to prevent formation of aggregates (approximately 1 h).

(iv) Rinse Hamilton syringe with ethanol, followed by autoclaved H_2O and HBSS between individual experiments. Sterilize outside by UV.

(v) **Preparation of solutions and Petri dishes.** For every carpet, prepare 100 µl solution in HBSS for each of the first and second stripes. For preparation of the first stripes, two solutions are required: one ephrin-A5-containing solution for the experimental situation (1. stripe: ephrin-A5-Fc versus 2. stripe: Fc) and one Fc-containing solution for the control setting (1. stripe: Fc versus 2. stripe: Fc). For the second stripes, only one solution containing Fc alone has to be prepared (see also controls in **Box 1**). First stripe solution: ephrin-A5-Fc or Fc, 10 µg ml^{-1}. Anti-human-IgG Fc-specific Cy3 conjugated, 2.5 µg ml^{-1}. Second stripe solution: Fc, 10 µg ml^{-1}. Anti-human-IgG Fc-specific, 2.5 µg ml^{-1}. For alternative concentrations, see refs. 33,36. The approximate active concentration when testing novel proteins is recommended to be within a range of 1–30 mg ml^{-1}. Spin down anti-human-IgG, Fc-specific antibodies (Cy3-conjugated and unconjugated) before use (1–2 min, 13,000*g*, 4 °C).

▲ CRITICAL STEP Without centrifugation, antibodies might produce aggregates resulting in unspecific guidance responses.

(vi) Preincubate first and second stripe solutions for 30 min under moderate agitation to allow for oligomerization of ephrinA5-Fc; keep solutions with Cy3-conjugated antibodies in the dark.

(vii) Prepare Petri dishes with matrices meanwhile by adding one matrix with channels facing downwards in the middle of a 60 mm bacterial Petri dish (see **Fig. 2b**). Label margins of stripes using a marker pen at the bottom of the dish (**Fig. 2c**).

▲ CRITICAL STEP Remove any air bubbles between matrix and dish surface, as this may cause leakage of solution.

(viii) **Preparation of the first set of stripes.** Prepare the first set of stripes by applying first stripe solution to the matrices. There are two possible methods: in the first (injection with a Hamilton syringe), insert the bent tip of a Hamilton syringe into the inlet channel of one matrix and inject slowly 100 ml of the first stripe solution (**Fig. 2d**). The solution should slowly pass the stripe channels and leave the matrix opposite to the inlet channel. In the second method (using a vacuum), add 100 µl of first stripe solution on outlet hole and suck solution through the channels by applying vacuum at inlet channel (**Fig. 2e**). It is critical to leave a small drop covering the outlet channel to prevent drying out.

(ix) Once all matrices of all dishes have been injected with first stripe solution, incubate all dishes for 30 min at 37 °C in a moist chamber to prevent drying out of stripes.

BOX 1 | CONTROLS IN STRIPE ASSAY EXPERIMENTS

Controls in the original version of the stripe assay

In **Figure 3c**, we illustrate the response of temporal and nasal axons in the original version of the stripe assay. Temporal and nasal axons exhibit differential sensitivity toward the repulsive activity of posterior tectal membranes, with temporal axons being repelled and nasal axons being insensitive, thus growing out in a random fashion. Therefore, in this kind of experiment, the ideal case of an internal control is provided.

If analyzing guidance properties of novel membrane fractions of different tissue sources, we recommend the following set of controls:

1. Prepare striped carpets with both stripes containing the same membrane fragments; this should result in random outgrowth.
2. To selectively inactivate the activity of membrane fractions under investigation, heat inactivation can be employed[1]. For example, by incubating membrane solutions at 63 °C for 8 min, all guidance activity can be eliminated[1].
3. The order of arranging membrane fractions into the sets of first and second stripes should be reversed; with this control, axons being, for example, repelled by membrane compounds in the set of first stripes (thus growing preferentially on the set of second stripes) should now—in the reverse order—be repelled by the (unlabeled) second stripes, thus growing preferentially on the labeled set of first stripes.
4. Reduce receptor–ligand interaction of axons with carpet molecules by exogenously adding purified receptors or ligands into the medium[36,38].

Controls in the modified version of the stripe assay

Typically, when preparing a set of carpets (e.g., 10–12), at least 3–4 carpets should be designated for the control experimental situation. Controls to be included in the modified version of the stripe assay are the following:

1. Prepare carpets with both sets of stripes containing control molecules (e.g., Fc versus Fc; see **Fig. 3e**). Also, carpets with both sets of stripes containing the molecule under investigation should randomize the guidance decision of axons (see also ref. 4).
2. Reverse the order of the sets of first and second stripes (see above).
3. Reduce receptor–ligand interaction of axons with carpet molecules by exogenously adding purified receptors or ligands into the medium (see above).

(x) Wash by slowly rinsing the matrices with 500 µl HBSS employing either the Hamilton syringe or the vacuum method (as described in Step viii).

(xi) Remove excess HBSS and matrix from the Petri dish by lifting the matrix starting at the upper right corner opposite to the injection channel (**Fig. 2f,g**).

(xii) **Preparation of the second set of stripes.** Immediately after removing the matrix, add 100 µl of second stripe solution to the previously labeled area of stripes (labeled in Step 1B(vii), also see **Fig. 2h**).
▲ CRITICAL STEP After removing the matrix of one dish, immediately add second stripe solution to prevent drying out of first stripes. Do one dish at a time.

(xiii) You may find it helpful to encircle the stripes with a Pap-pen (approximately 2 cm in diameter; **Fig. 2h**) to reduce the total volume of cell culture medium needed; however, this is an optional step.

(xiv) Incubate all dishes for 30 min at 37 °C.

(xv) Remove second stripe solution as much as possible but not completely to prevent drying out of the carpet and wash once with HBSS.

(xvi) Incubate stripes with 100 µl laminin in HBSS for 2–3 h at 37 °C (20 µg ml^{-1}). Laminin provides an overall outgrowth-promoting substrate, which is necessary, particularly when testing repulsive molecules; laminin incubation might be skipped depending on the molecule under investigation. Also other extracellular matrix components such as fibronectin or collagen can be tried. During laminin incubation, prepare cells to be examined. Note that this step is optional and might be omitted while testing novel proteins for their guidance activity.

(xvii) Wash stripes once with HBSS.

(xviii) Add 500 µl culture medium and keep dishes in the incubator until cells are ready to be plated.

Plating of cells and explants on carpets

2| For plating dissociated single cells (e.g., mouse hippocampal neurons), spread 10^4 cells per stripe area. Mouse postnatal hippocampal neurons can be prepared according to a previously published protocol[37]. When using explants (e.g., dorsal root ganglia, vomeronasal organ, *Xenopus* or zebrafish retina), distribute 10–20 explants on one carpet. To allow radial outgrowth of nerve fibers from the explant, sharp tungsten needles should be employed to cut tissue into squares or rectangles (average diameter 300–500 mm). Alternatively, as in the case of chick RGC explants (see ref. 2), E6 retinae can be flat-mounted on nitrocellulose filters and cut into 200–300 mm wide strips using a tissue chopper. Two of these retinal explants can be arranged on one carpet.

3| Incubate cultures for 24–48 h. If desired, perform standard immunocytochemistry employing, for example, a tubulin-specific antiserum or fluorescent labeling of RGCs with DiI[2].
? TROUBLESHOOTING

● **TIMING**

To prepare 10–12 carpets either by the original or by the modified version of the stripe assay, it takes approximately 4–5 h.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Solution
No or fuzzy white intermediate phase in the sucrose gradient	Amount of starting material too low or high	Use 40–60 mg of tissue as starting material
	Generally, membranes derived from transfected tissue culture cells tend to result in less sharp intermediate phases	For tissue culture cells, use four dishes of 10-cm diameter with approximately 1.5–3 × 10^6 cells for each of transfected cells and parental cell line as starting material
Poor outgrowth of axons	Pap-pen might be cytotoxic	Skip or try a different one from another company
	Membranes/proteins used are poor substrates for cell motility	Try to add laminin or other growth-promoting substrates (e.g., fibronectin)
		Reduce the concentration of membranes or purified proteins
No guidance decision	Proteins in membrane fractions might be degraded	Add protease inhibitor freshly to HB
		Work quickly through preparation procedure
		Do not store membrane fractions for too long (>2–3 months) and measure activity again

TABLE 1 | Troubleshooting table (continued).

Problem	Possible reason	Solution
	Lot of commercially available purified proteins does not work	Check lot of purified proteins on, for example, SDS-PAGE or in collapse assay
	Molecules do not adhere to surface	Check with an antibody directed against the molecule for adherence to surface
	Molecules do not function as guidance cue in substrate-bound form or not at all	Try different guidance tests suitable for secreted molecules (e.g., collagen co-culture assay, turning assay)
Stripe pattern is weak, stripe borders are not sharp	In modified stripe assay:	Make sure no air bubbles are between matrix and dish
	Matrix did not adhere to dish evenly and firmly	Matrices have to be completely dry before use
	Matrix dried out	Clean matrices after 2–3 experiments by removing, for example, dust with transparent tape from striped surface
	In original stripe assay:	Have second stripe solution already at hand when removing matrix
	Nucleopore membrane did not cover stripe margins	
	Nucleopore filter dried out	Make sure nucleopore filter overlaps the margins of white and blue matrices; cut larger nucleopore filter pieces
		Make sure nucleopore membranes are arranged in the right orientation (cut left top corner; shiny side of membrane up) onto suction filter
		Work quickly, transfer nucleopore membranes rapidly on prepared dishes with PBS drop between sucking steps
Unspecific guidance decision in control stripe assay (mostly in modified stripe assays)	Drying out of first stripe pattern leads to physical barriers at stripe borders	Work quickly through the protocol
	Anti-human IgG, fc-specific antibody aggregated	Check the quality of stripes under fluorescence before use
		Spin down antibody (1–2 min, 13,000*g*, 4 °C)
		Reduce the concentration of purified protein and control protein; vary the level of 'clustering' (add less anti-Fc antibody)
	Serum (e.g., different lot) used for growth medium	Use serum-free medium
		Try various serum batches

ANTICIPATED RESULTS

Images depicting typical anticipated results are given in **Figure 3**. **Figure 3a** shows the typical pattern of alternating stripes derived from membrane fractions of the anterior and the posterior chicken optic tectum, respectively. Posterior membranes are labeled with rhodamin-coupled beads. **Figure 3b** shows alternating stripes consisting of purified ephrin-A5-Fc (labeled with FITC-conjugated anti-human IgG, Fc-specific antibodies) versus Fc alone (dark stripes). Note that stripe borders using the modified version of stripe assay appear sharper compared to the original version (see **Fig. 3a**). The circle indicates the position and size of an explant cultured on top of the carpet. **Figure 3c** shows a stripe assay experiment using alternating stripes of anterior and posterior tectal membranes (original version), cultured with RGC explants (labeled in green). Axons of the temporal retina are strongly repelled by cues present in the posterior membranes (labeled in red). Nasal axons growing in a random fashion serve as an internal control in this type of experiment. **Figure 3d** shows a stripe assay experiment using purified ephrin-A5-Fc (modified version). Ephrin-A5-Fc-containing stripes (in red) are alternating with Fc-only-containing stripes (not marked). In this setup, both temporal and nasal RGC axons (in green) are repelled by ephrin-A5-Fc, thus growing preferentially on Fc-containing stripes. Note that owing to fasciculation, not the entire stripe width is occupied by RGC axons.

Figure 3e shows a control stripe assay experiment using carpets with both stripes consisting of Fc alone. This important control—to be included in every stripe assay experiment—reveals unbiased random outgrowth of axons.

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