Cell Behavioral Responses to Eph/ephrin Signaling in Primary Migrating Neural Crest Cells

Eph/ephrin signaling is known to be important during many embryonic processes, and is of particular importance to the migration of neural crest cells. To better understand the exact role of Eph/ephrin signaling in neural crest cell migration, we developed a novel cell migration assay to study the early cell behavioral responses of migrating primary avian neural crest cells to focal stimulation with ephrin-B ligands. We developed protocols for derivatizing small glass beads with ephrin-B proteins and for presenting these beads to migrating neural crest cells in culture, in a spatially and temporally defined manner, using optical tweezers. We continuously imaged the cells before, during, and following contact with ephrin-B beads and observed increased severity and frequency of lamellapodial collapse and cellular polarity change in response to ephrin-B1 beads when compared to beads coated in Protein-G.

We concluded from these experiments that the primary reaction of neural crest cells to ephrin stimulus is to change the directionality/polarity of the cell, rather than a global collapse of all projections, as has been previously reported [1, 2]. This polarity change occurs quickly (1-4 minutes) and as such is unlikely to involve transcription or translation, though these mechanisms were not explicitly ruled out. Additionally cells reacted more strongly to beads presented on their leading edges than their lateral surfaces and a cluster of beads presented simultaneously elicited a stronger effect than a single bead. Cells also proved capable of responding to multiple sequential stimuli, even when presented only minutes apart. We close by discussing the strengths and weaknesses of this assay.

Andrew J. Ewald and Scott E. Fraser

Biological Imaging Center

California Institute of Technology

Introduction

Migrating neural crest cells have been shown in published reports [1, 2] and in research for this thesis (Chapter 2) to be repelled by ephrin-B1. The published reports showed that, at the population level, migrating neural crest cells avoid regions of a *in vitro* substrate that contain more ephrin-B1. However, their assay was unable to address the mechanism or kinetics of this repulsion in detail. Furthermore there was significant observed variability in the response of individual neural crest cells within their assay. In Chapter 2 we attempted to address whether this heterogeneity in response was due to some inherent heterogeneity in the neural crest cells themselves or an artifactual variability caused by unintended systematic variations in the substrate pattern of ephrin-B1. To address that question I improved the neural crest cultures to more reliably produce similar cells, identified serum free culture conditions, improved the imaging conditions to better resolve cellular responses to the patterns, and significantly improved the substrate patterning techniques to produce more reliable patterns of ephrin-B1. The variability persisted and was so great as to prevent a meaningful analysis of single cell behavior.

We then focused on one remaining source of experimental variation inherent to the stripe assay: since we could not control when or how often the neural crest cells encountered the ephrin-B1 regions of the substrate each neural crest cell experienced the experiment differently. Some crest cells likely never encountered the ephrin regions at all, some migrated out onto the stripes directly and may have adapted to their presence, and only an unpredictable and small subset encountered the stimulus for the

first time while we were watching. For these reasons I came to believe that the stripe assay, whether implemented via adsorbed or photolithographed proteins, was intrinsically an assay for measuring the average response of a population of cells to a guidance cue and was not suited to detailed single cell analysis of the kinetics and nature of ephrin-B induced changes in cell behavior.

I instead devised a new, complementary assay for focusing on these questions. By derivatizing glass beads with ephrin-B1 and then using optical tweezers to manipulate the beads into contact with the cell, I sought to strip away most of the variability in the cell's experiences within each experiment. Optical tweezers are a particularly good tool for presenting beads to cells as their maximum force output is significantly less than the maximum force a cell can exert [3]. The forces exerted by an optical trap on a cell are small from a cellular perspective, while alternate modes of bead presentation, such as mechanical presentation with a glass micropipette, are capable of imparting much larger forces. Each neural crest cell migrated unrestricted on a fibronectin coated glass coverslip until the moment that it was introduced to an ephrin coated bead. By controlling the exact timing of first onset of the stimulus we sought to measure the early cell behavioral response of primary migrating neural crest cells to ephrin-B signaling.

Conceptually analogous experiments have been done through solution addition of EphB receptors to retinal axons [4] in a modified growth cone collapse assay, and the effect of solution addition of EphB receptors on the cytoskeleton of NG108 cells was monitored at 1 hour after stimulation in another report [5]. In the first report growth cones were

observed to collapse in response to EphB receptors and in the second the actin cytoskeleton was reorganized in response to soluble EphB receptors. Our experiments are complementary to these as ours were conducted in primary neural crest cells, with addition of surface bound ephrins, rather than Eph receptors. Both the Eph “receptors” and the ephrin “ligands” are capable of transducing signals into the cell upon which they are expressed, so it is interesting to study both directions of stimulus. There is some recent evidence that the stimulation of Eph expressing cells with ephrin ligand is the relevant signaling pathway for neural crest migration (as opposed to stimulation of ephrin expressing cells with Eph receptor), as the cytoplasmic domain of ephrin-B2 was shown to be dispensable for cranial neural crest migration in a mouse knockout [6].

We chose to focus our efforts on the *in vitro* study of neural crest cells in order to maximize our control over the cell’s microenvironment. We feel that it is of particular relevance that we are using primary neural crest cells, within hours of explanting from the embryo, as these cells normally see and respond to ephrin-B ligands and should maintain fairly normal gene expression over this time period. Such studies are particularly useful for examining the role of ephrin signaling in guiding the neural crest, because ephrin-B knockouts in mouse are difficult to assay for neural crest migration defects, due to a pronounced cardiovascular defect [6-10]. It is also worth highlighting the results of a recent paper [11] that demonstrated a repulsive role ephrin-B signaling for early migrating trunk neural crest cells and a migration promoting role for ephrin-B signaling with late migrating neural crest cells. This dual role for ephrin signaling, which depends on cellular context, is consistent with published studies in other systems that

note that ephrin signaling can switch between mediating repulsive and adhesive interactions [12, 13].

We chose to focus our efforts with this new optical tweezers based migration assay on two specific areas: the nature and timing of the early cell behavioral response to ephrin-B stimulus, and the differential sensitivity of different regions of the neural crest cell to stimulation. These two questions take best advantage of the optical tweezers ability to precisely control the spatial and temporal presentation of ephrin protein. This specific control is missing in solution addition experiments where proteins are applied to the bath. It is also worth noting that the tight binding of protein to the glass beads makes the ephrin stimulus intrinsically directional (one part of the cell sees the bead and the rest doesn't) in a way that is difficult to mimic with a solution addition of protein. The key aspect of the timing that we sought to address was the delay between observing a cell migrating onto a stripe and observing it collapsing in response to a stripe [1]. This delay was reported to be between 20-40 minutes and we originally sought to determine the nature of the delay by comparing the effect of transitory contact between the bead and cell and persistent contact. This proved unnecessary for two reasons: first the cells tended to irreversibly pull the beads out of the trap and second the cells reacted much quicker than previously reported, typically 1-5 minutes after first contact.

Materials and Methods:

A. Laser Tweezers

Optical tweezers work by tightly focusing a beam of light and then using the focus of that beam to apply force to small objects. When the gradient force directed towards the focus of the laser is greater than the scattering force of the laser beam in the direction of the beam, then it becomes possible to stably hold a small particle in the center of the trap [14]. The gradient force is associated with changes in the momentum of light as it reflects and refracts off the dielectric interface of the glass bead and water [15]. For our experiments we used the optical tweezers facility of the Molecular Materials Research Center of the Beckmann Institute at Caltech. Figure 1 shows an image of the optical tweezers set-up and identifies the major components. Briefly, as depicted in Figure 1, the light from a B&W TEK BWR-1500 Nd:YVO4 infrared laser passes through a telescope (optics which expand then focus the beam), through a beam expander, and into a prism which splits the light into two separate beams, each of which is directed onto the surface of an adjustable mirror. The beams then recombine at a second prism, pass through a focusing lens and are directed into the Keller port of a Zeiss Axiovert S100 TV microscope with a 40x Plan Neofluar Phase 3 oil immersion objective. As a result of the prism splitting the beam to two independently adjustable mirrors, there are two independently adjustable optical traps built into the system. Both the mirrors and the stage are manually controlled, which required careful manipulation to avoid jerking the bead out of the trap. With practice, the system provided good trapping power and good control of the beads. The one caveat to the system is that it does not provide full

automatic laser safety protection. A supplemental infrared blocking filter was installed internally within the Axiovert microscope, but it should not be counted on to provide complete protection from focused IR light.

Microscopy

We continuously imaged the cells using Phase 3 contrast on a Zeiss Axiovert S100 TV microscope with a 40x Plan Neofluar Phase 3 oil objective lens. Images were collected by a Hamamatsu C5985 CCD camera and digitized by a Flashbus frame grabber card and acquired in the Metavue software program (Universal Imaging Corporation). The system provided good contrast on the details of lamellapodial and filopodial dynamics, without the need for any exogenous contrast. We were capable of acquiring 640 x 480 pixel tiff files at video rate, but this produced too many large files (1800 per minute). Instead we collected at 1 frame per second. This proved sufficient, and for many analysis steps we only examined every 10th frame, as a 30 minute movie at 1 frame per second yielded 1800 frames. Image processing was done in Adobe Photoshop 7.0 and Image J.

Functionalization of Glass Microspheres

For these experiments, we used recombinant mouse ephrin-B1 (R&D Systems), expressed as a fusion with the FC domain. We tethered this to glass beads via a biotinylated Protein-G (Calbiochem), that was linked to the bead via streptavidin coupled to a biotinylated alkyl silane self assembled monolayer. We decided on this somewhat indirect coupling approach based on previous reports that the function of

ephrin-B ligands within *in vitro* assays was dependent on the oligomeric state of the ligand, with clustered ligands being more active [16]. Our method should provide a series of strong attachments that, combined with washing after ephrin-B1 treatment, couple the proteins strongly to the beads. Thus we would expect that the bead is not a source of diffusing ephrin-B1, but rather only a contact mediated source. This is consistent with our experimental observations that cells do not appear affected by beads that they are not in contact with. Our detailed protocol for derivatizing glass beads is presented in A2-11, with full details of suppliers and part numbers for all components. That protocol is based on a protocol originally developed by Kevin Thigpen for other purposes.

Neural Crest Cell Preparations

Trunk neural crest explants were performed in general accordance with standard methods [17]. Our neural tube explant protocol was optimized as described in Chapter 2 and is presented in detail with suppliers and part numbers for all components in A2-6. Briefly 44-52 hour quail embryos were surgically extracted, the trunk segments were mechanically isolated, and the neural tube were enzymatically separated from the surrounding tissues with dispase II. The neural tubes were isolated from the attached notochord and rinsed through serum containing media to quench the dispase. They were then explanted onto glass substrates treated with human fibronectin. The typical culture media was F12 basal media supplemented with N2 protein supplement, penicillin and streptomycin. For imaging, the media was supplemented with 20 mM Hepes buffer.

Fibronectin Substrates

The glass substrates for these experiments were 40 mm coverslips (BiopTechs), which were coated with an alkyl silane layer and then treated with fibronectin, as described in A2-13. They should have been very uniform from batch to batch and region to region.

Culture Chambers

We used a Bioptechs closed FCS2 chamber, with integral heating and perfusion. The temperature was maintained at 37° C and fresh media was perfused at a slow rate into the chamber. Beads were added using a one milliliter syringe connected via a T-junction in the tubing just before the entry point the chamber. Beads were added to a flowing stream of media. This approach yielded reasonable, but not complete, control over the timing and amount of bead addition. It was typically necessary to clamp the exit hosing whenever adjusting the connection on the syringe, to prevent air bubbles being sucked through the chamber. Likewise, it was typically necessary to mix up the beads in the syringe prior to injection. Sometimes the beads would clump up into small clusters of 2-10 beads, for unknown reasons.

Basic Protocol

The timing of a typical optical tweezers experiment on neural crest cells is presented in A2-9. The details of the actual migration assay were as follows. At the end of day 3, I explanted neural tubes onto fibronectin substrates. First thing the next morning I verified that neural crest cells had emigrated out and mechanically removed the neural

tubes with forceps. I then loaded a coverslip with cells into the FCS2 chamber, took it to the tweezers room and loaded it into the set-up. I then typically allowed 30 minutes for the cells to equilibrate to their new surroundings. Once the culture had adapted I selected an isolated cell and begin a 30 minute time-lapse, collecting a frame every second. Once I had a couple minutes of images of that cell I pulsed the beads into the chamber. It often took a few seconds to a minute or two for the beads to work their way into the field of view. I then captured a bead and presented it to the cell, typically on the leading edge. Since the cell could pull the bead out of the trap, and to prevent heating up the cell, I then turned off the trap and simply watched the cell's behavior from that point for a total of 30 minutes. Occasionally a second bead would be trapped and introduced to the cell following the first stimulation.

Data Analysis

Each of the 30 minute movies (1800 frames) was reduced to a 180 frames by selecting every 10th frame using Image J. I then applied a Sharpen More filter and a Northeast Shadows filter to the stacks in Image J and saved the stacks out as .avi files for ease of use across computers. Initial scoring was to attempt to blindly identify control movies (Protein-G beads), from experimental movies (ephrin-B1). I scored them and was unable to reliably, blindly classify all of the movies as either control or experimental. Katy McCabe also agreed to blindly score all of the movies to determine which ones were control and which ones were experimental. She was also unable to do so accurately and similarly made errors of all types: incorrect classification of control as experimental and experimental as control as well as correct identifications. I concluded

from our inability to reliably distinguish control from experimental movies that all categories of cell behaviors were present in both conditions. To address whether there was a difference in frequency or severity of response I scored each movie for the number of major collapses per movie (30 minutes), the number of polarity changes per 30 minutes, and a score of 0-2 for overall reaction. If the cell appeared indifferent to the bead I assigned it a score of 0. Mildly repulsed was 1 and strongly repulsed was 2. David Koos also scored them blind. He systematically assigned lower scores than I did, but my low scores received low scores from him and his high scores received high scores from me. There were observed differences between control and experimental in the scoring of both David Koos and I.

Results

Optical Tweezers Migration Assay Works

All of the necessary components for the migration assay: primary cell culture, bead derivatization, bead perfusion, temperature control, bead capture, bead presentation to cells, and image collection and analysis all worked well as a synchronized assay. It was routinely possible to perform 8-10 separate bead experiments in a day, with 2 days of preparation. Some of this preparation would be suitable for more than one day of experiments, so in a long week 20-30 movies experiments could, in principle, be collected. Since these are intrinsically single-cell experiments accumulating significant numbers still takes some time. However, the key is that enough experiments can be run in a day to test multiple conditions (e.g., Protein-G and ephrin-B1) and reliably get

multiple examples of each one, even allowing for some experiments not going as planned.

Interpretability

One unanticipated limitation in the assay, that only emerged during data analysis, was that it was very difficult to interpret the behavior of cells that were already contracted, were not highly polarized, or were in contact with other cells. The cell that we were forced to look for was a highly polarized, highly flattened, migratory cell that was isolated from its neighbors. These cells were not typical of most in the dish. The underlying reason for this bias is that it was very difficult to interpret if a cell with no obvious polarity, or with most of its projections already retracted, had been repulsed by the bead or was simply maintaining a pre-existing semi-collapsed state. Since we were scoring for changes in cell behavior or cell polarity, it was necessary for the cell to have an obvious polarity or baseline level of protrusive activity at the beginning of the assay. Cells in contact with their neighbors will be addressed later in this analysis.

Types of Experiments Run

There were three major types of experiments run on neural crest cells. The first and most generally informative were leading edge stimulations, where a polarized cell was hit on its currently most active lamellapodia with one or more beads. To test whether there were regional differences in cellular response, some neural crest cells were instead contacted on a lateral surface distant from the leading edge. Finally to query

the ability of neural crest cells to process multiple stimulations some neural crest cells were serially or simultaneously contacted with several beads.

Reaction of Cells Was Intrinsic to Beads, Not to Laser Trap

We interpret the cells' reactions in these experiments to be primarily due to some direct influence of the bead, rather than the laser trap for two reasons, both unplanned. For both it is important to remember that beads are added by perfusing a pulse of them into the culture chamber. As such we had only partial control over where the beads went and some beads would land near cells through no conscious choice of our own. Since there were sometimes surplus beads in the cellular environment, cells could encounter beads on the surface that had never been manipulated with the laser. In multiple independent experiments cells acquired beads in this manner and reacted indistinguishably from cells that encountered beads from the laser trap. As further evidence occasionally cells would lose contact with a bead that they were repulsively reacting to. Their repulsive reaction would quickly cease after losing contact with the bead and they would assume a neutral polarity.

Difference in Degree and Frequency, Not in Category of Cell Behaviors

Our primary control for the specificity of the interaction between the cell and the bead was to contrast the cellular response to a bead coated in ephrin-B1 via Protein-G and a bead that was prepared identically but simply never exposed to Protein-G. There were no categorical differences in cell behavior between the two conditions, in that blind

scoring did not identify cell behaviors that were present in the ephrin-B1 experiments, but not in the Protein-G experiments (as independently and blindly scored by Andrew Ewald an Katy McCabe). Categorical differences would obviously have been desirable. Instead we developed three metrics and tested for differences in degree or frequency of reaction. We counted for number of polarity/directionality changes per 30 minutes, number of major retractions/collapses per 30 minutes (Figure 3-2), and degree of reaction (Figure 3-3): 0 for neutral, 1 for mildly repulsed by bead, and 2 for strongly repulsed by bead. Differences existed in all three categories. The detailed scoring for all analyzed movies is reported in Appendix 2. Cells contacting ephrin-B1 beads collapse more often, changed their cellular polarity more often, and responded more severely to ephrin-B1 beads (Figure 3-3). It is worth noting that cells in both conditions demonstrated a high degree of protrusive activity, making all of these judgments somewhat difficult. It is also worth noting that cells responded more dramatically to a cluster of ephrin-B beads than to a single bead.

As a more objective test of the noted differences, a second blind evaluation was made of all of the movies by David Koos. These scores were systematically lower than those of Andrew Ewald, but the high Koos scores were high Ewald scores and the low Ewald scores were low Koos scores. The details of the two sets of scores are presented in Appendix 2 and the conclusions are summarized in Figure 3-4. Both sets of scores reveal a marked difference between Protein-G and ephrin-B1 experiments.

Typical Interaction of Cell with Beads

In the majority of cases, with both ephrin-B1 and Protein-G beads, the cell would take the bead out of trap and retain it with sufficient force that the trap could not take it back. Most beads that were captured in this manner were retrogradely transported and they were rarely released. Due to this unexpected phenomenon we were unable to directly test the differences in cellular response to transient vs. sustained contact with ephrin-B1 beads. Most neural crest cells that were going to exhibit a response to ephrin-B beads had done so within 1-4 minutes from first contact. A good example of a rapid reorganization is Figure 3-5. Within 1 minute of contact with an ephrin-B1 bead on its leading edge the experimental cell has begun to reverse its polarity and by four minutes it has retracted its previously dominant lamellapodia and extended a new one in the opposite direction. This reversal of polarity is the prototypical strong reaction of a polarized neural crest cell to ephrin-B1 beads. Figure 3-6 presents a similar case: the cell in question is migrating towards the bottom of the frame until it is contacted with a cluster of ephrin-B beads. It takes the cluster of beads, transports them towards the nucleus, collapses its proximal lamellapodia, and extends a new leading edge on the opposite side of the cell.

Contact with the lateral edge of a neural crest cell with ephrin-B1 beads was sufficient in some cases to dramatically reorganize the cells polarity, as seen in Figure 3-7, but this was not typical. In general the cellular reaction to lateral edge ephrin-B1 presentation was less dramatic. Figure 3-8 presents the relative proportion of repulsion scores for lateral versus leading edge presentation. We conclude from these limited data that

neural crest cells are more responsive to ephrin-B signaling on the leading edge of the cell. This graph also serves to highlight the differential response that only subtly different modes of ephrin-B presentation can evoke. It also highlights that our pooled averages for the experiments are averaging across different experimental designs which evoked different responses of differing severity.

Figure 3-9 highlights a highly polarized cell that has received two sequential ephrin-B stimuli on its leading edge. It responds by to the first by bifurcating its broad lamellapodia and briefly assuming a bipodal shape, before collapsing the lamellapodia near the site of the second contact and migrating away. This experiment demonstrates the ability of a neural crest cell to sequentially respond to two stimuli over a span of less than a minute. Interestingly, the response is also sequential, not additive, implying that the cell either remained sensitive or quickly regained sensitivity to ephrin-B stimuli very shortly after the first contact. These sorts of details are only accessible via time-lapse microscopy.

The examples cited in figures so far were all strong (score=2) reactions to ephrin-B1. To give a fuller sense of the range of response, Figure 3-10 presents a neural crest cell that reacts mildly to ephrin-B1 stimulus (score=1 AJE, 0 DK). This movie also highlights the difficulty in scoring the response of cells without obvious directional polarity or large leading edge lamellapodia. The fractional loss of adherent surface area in this case may well be more than in Figure 3-9, but it is more difficult to determine the nature of its response. Figure 3-11 presents a polarized neural crest cell that was presented with a

Protein-G bead on its most active lamellapodia. This lamellapodia collapses nearly completely and there are also several small retraction events coincident with transient contact between the cell and beads on the surface (Score=2 AJE, DK). Finally Figure 3-12 shows a mild reaction to a Protein-G bead. The global shift from the first to second frame is due to a shift in the position of the stage, not cell movement. The cell is already somewhat retracted so it is a bit difficult to score, but the lamellapodia in contact with the bead retracts noticeably (Score=1 AJE, 0 DK).

Discussion

Conclusions

We conclude from these experiments that the normal response of a neural crest cell to ephrin-B ligand is to quickly (1-4 minutes) retract local lamellapodia coincident with a global increase in protrusive activity elsewhere. In cases where the cell maintains contact with the bead that region of the cell tends to remain collapsed, and the cell typically migrates in the opposite direction. This response is different than the 20-40 minute delay then global collapse that was previously reported [1]. We attribute the differences in timing to the fact that our assay enables more accurate determination of the time of first contact, and possibly to the fact that a bead coated in ephrin protein may be a more intense signal to the cell, due to its very high local concentration. The leading edge of the cell was more sensitive to ephrin-B stimulus than the lateral edge, and clusters of beads elicited a stronger response than individual beads. These conclusions are consistent with recent reports of migrating neural crest cells in the

hindbrain of chick embryos: neural crest cells migrate in a directional manner, with few undergoing prolonged global collapses in normal embryos [18-20].

Level of Confidence in Conclusions

We are not unambiguously certain of the above conclusions. The differences between the Protein-G and ephrin-B1 conditions, while present, are not overwhelming. The movies of neural crest cells strongly reacting to ephrin-B1, taken on their own, while highly suggestive about the kinetics and mechanism of the cell behavioral changes in neural crest migration induced by ephrin-B signaling, can not be considered compelling without further experimental support. There are two basic problems. The first is that there is a considerable variability in the response within a given condition. The second is that the Protein-G beads evoke a strong reaction, in a subset of experiments.

Variability

Our most likely explanation for the variability in the response of neural crest cells to ephrin-B beads is intrinsic biological differences between cells, as we have controlled nearly every other aspect of the experiments. A recent paper supports this idea, as they noticed that early migrating neural crest cells are repelled by ephrin-B1 and late migrating cells perceive ephrin-B signal as migration promoting [11]. One way to address this concern is to more rigorously restrict the types of neural crest cells in the culture, by experimentally isolating only early or late neural migrating neural crest cells and then contrasting their response to ephrin-B1 beads. Another possibility is that

there is a threshold effect, whereby a certain amount of ephrin-B stimulus is required to elicit a reaction. This might vary from cell to cell based on receptor expression level. There is some support for this idea in our observation that clusters of beads elicited stronger reactions than single beads.

More troubling is the variability in the response of cells to Protein-G beads. I can think of two likely explanations. The first is that the background level of protrusive activity, in isolated neural crest cells *in vitro*, is so high that our interpretations of cause and effect are suspect. Visual inspection of cultures and long-term time-lapse data on neural crest cell cultures provides some support for this idea. I have noted that the “quietest” cells in a culture are those in extensive contact with their neighbors; isolated highly polarized cells tend to collapse and extend dramatically even in the absence of obvious guidance information. Another possibility, of greater concern, is that the cellular reaction has nothing to do with ephrin signaling and instead is a response to the Protein-G, streptavidin, or bead chemistry. The coupling proteins should be innocuous to neural crest cells, but this possibility can not be excluded. Alternately the cell could be reacting through some more basal cell adhesion mechanism. Arguing against this idea are studies of cellular reactions to fibronectin coated beads, presented with optical tweezers, in which highly local responses (8-12 um2) to the bead were observed [3]. In contrast we are definitely observing global responses throughout the cell.

Possibilities for Improvement

The next direction for this project, to identify the best future assays modifications, would be to simply conduct more leading edge neural crest experiments. We expected that we would gain significantly more control over a cell's experience in this assay, as contrasted with the stripe assay (Chapter 2), and we did. However, Figure 8 demonstrates that cells contacted with ephrin-B beads on different parts of their circumference react differently This is an interesting observation, but it means that tighter control needs to be exercised on the type of experiment being conducted (leading or lateral edge, etc.) and that serendipitous experimental variation (i.e. cell contact with other cells or additional beads) needs to be excluded from the core analysis. In practice this means performing significantly more migration experiments. Another avenue worth considering is to alter the coupling chemistry used to attach the protein to the bead. The possibility still exists that some variation of experimental technique will cause a subset of the neural crest behavior to become more prominent or disappear, and thereby increase our confidence that the dynamics we observe are specific.

We could achieve a qualitative improvement in interpretability if we could visualize the activation of Eph signaling in the neural crest cell, while monitoring its morphology. The problem with monitoring cell morphology alone is that it is very difficult to be certain when, and in which cells, Eph signaling has been activated. This variability alone could explain all the differences within each condition; the cells that responded strongly to ephrin-B1 beads may be the only cells in which Eph signaling was activated. It could

also let us directly assay for the necessary threshold of ephrin contact required for Eph signaling. At present we are not aware of any dynamic means of monitoring Eph signaling in living cells.

Posthoc Evaluation of Experimental Design

Having completed this project, I have had time to consider the quality of the experimental design and the return of biological conclusions for time invested. I developed and pursued this assay consciously aware that it was a high-risk high-reward undertaking. If neural crest cells responded in a more consistently different way to ephrin-B1 beads than Protein-G, and if their background level of cell behavior in isolation was lower, I still believe that this experimental paradigm would be a powerful way to evaluate the immediate early response of neural crest cells to Eph/ephrin signaling. Once those clear behavioral responses emerged, there are many experimental possibilities, including testing the effect of different mutations on the ability of the resulting protein to evoke the characteristic response. The problem is that unless a clear, repeatable characteristic response exists (which is unknowable until tested), the assay produces no firm conclusions. A related problem with this type of experiment is that is was very labor intensive, taking a full year to experimentally implement, and months to acquire and interpret the data. Simpler, faster assays have answered many of the questions we sought to address [4, 5, 11]. I still think the optical tweezers migration assay was a well-designed experiment, but in the future I will make a faster determination whether a high-risk assay seems like it will be productive or not. I will

also pay more attention to whether the same questions can be answered via technically simpler means.

One element that I feel was missing from this experimental design that I will seek to correct in the future is autonomous cellular behavior. The central problem with the interpretation of the time-lapse movies in this project was that there was no biologically known cell behavior that was preserved in our neural crest cultures. By way of contrast explants of the dorsal marginal zone of a frog gastrula are known to autonomously execute their normal convergent extension cell movements even when surgically isolated and cultured [21]. Because there was no normal cell behavior preserved in our assay, the only regularity in cellular response would have to come from a regular response to our applied stimulus. A simple experimental variation would be to assay for delamination of neural crest cells from the neural tube in culture and test for the role of different signaling pathways in that decision.

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Figure 1: Panel A shows the optical tweezers apparatus in the Molecular Materials Research Center. As there are a number of extraneous components on the optical table, Panel B shows the core optics of an optical tweezers set-up. For simplicity several components in the optical path that are not critical to its performance are neglected in the schematic. The light from an infrared laser is steered into a telescope that expands and condenses the beam onto a second mirror which directs the light through an aperture to a prism. The first prism splits the light into two independent paths, which are directed to two independently controlled steering mirrors. A second prism then recombines these two beams and directs them through a focusing lens, and onto a final mirror that directs the light into the Keller port on the bottom of the microscope.

Figure 2: Three different experimental conditions were compared for the number of polarity changes and number of major collapse/retractions per 30 minutes (the time of a typical experiment). The control condition was neural crest cells cultured on the same microscope with no laser trap and no beads. This baseline condition was compared to cells exposed to beads coated in either Protein-G or ephrin-B1. The plot is expressed as a percentage representation of each score within each condition. Protein-G was less responsive than ephrin-B1 according to both metrics, but both were more different from control than from each other.

Figure 3: The overall reaction of the cell to the bead was characterized as a single score, with 0 being neutral, 1 being mildly repulsive, and 2 being strongly repulsive.

Cells exposed to ephrin-B1 beads reacted more strongly overall. When cells that made contact with other cells through the course of the experiment were excluded, this difference became greater.

Figure 4: Two different independent scientists scored the overall responsiveness of the cells to the beads, with 0 being neutral, 1 being mildly repulsive, and 2 being strongly repulsive. One scorer (Koos) consistently scored lower than the other (Ewald), but both observed a difference between ephrin-B1 and Protein-G beads, with ephrin-B1 eliciting a stronger reaction in both cases.

Figure 5: Two cells were filmed for 30 minutes. The cell on the right was contacted with a bead on its leading edge at t=0, while strongly polarized towards the top of the frame. This polarity is reversed substantially within 1 minute and completely by four minutes. The cell loses contact with the bead and then assumes a neutral polarity. This is an example of a strong reaction to the bead and was scored a 2 by AJE.

Figure 6: One cell was migrating towards the bottom of the field and was contacted with a cluster of ephrin-B1 beads on its leading edge. Within eight minutes it completely reorganized its orientation and began migrating towards the top of the field. This is an example of a strong reaction to the bead and was scored a 2 by AJE.

Figure 7: An example of lateral edge presentation of ephrin-B1 beads to a neural crest cell. Within 4 minutes of contact the cell had withdrawn its most active lamellapodia and

by eight minutes it had reorganized to migrate in the opposite direction from its original orientation. This was considered a strong reaction to lateral presentation and was scored a 2 by AJE and DK.

Figure 8: The repulsion scores for leading edge and lateral edge experiments were separated from the totals and expressed as a percentage of the scores representation in the dataset. The differences between ephrin-B1 and Protein-G are not as dramatic in either case, but it is clear that the cell responds more strongly to contact on its leading edge than its lateral surfaces.

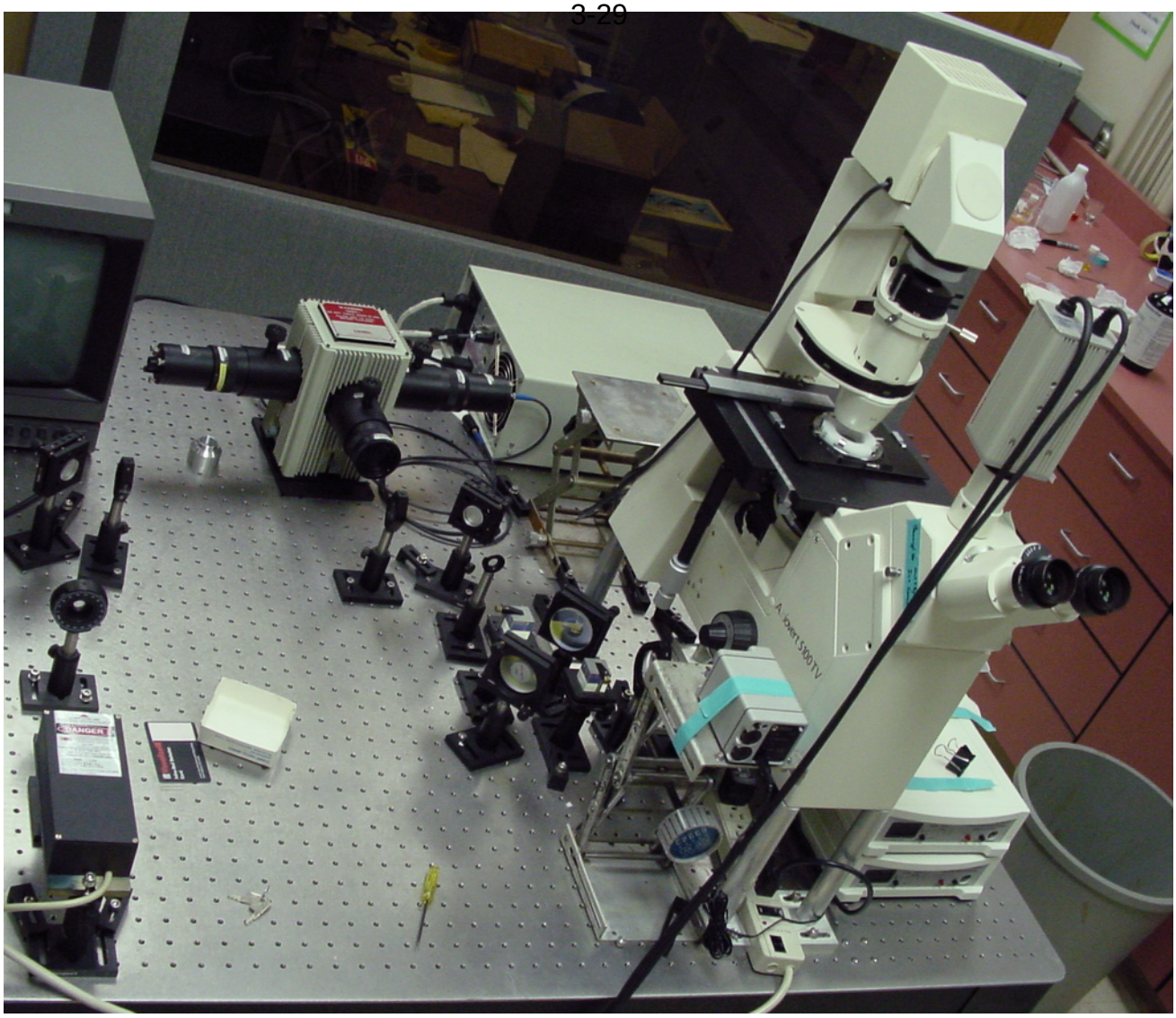
Figure 9: This cell was actively migrating towards the bottom of the frame and was organized with one broad downward pointing lamellapodia. The cell was contacted twice in rapid succession (less than 1 minute gap) on its leading edge with ephrin-B1 beads. The reaction was similarly sequential. First the broad lamellapodia collapsed in the vicinity of the first contact and the cell appeared bipodal. Then the area around the second contact collapsed and the cell began migrating away at right angles to its previous direction. This was considered a strong reaction and was scored a 2 by AJE.

Figure 10: This cell was not actively migrating and did not have an obvious directional polarity. It was contacted with an ephrin-B1 bead on one of its three projections. The cell contracted that projection modestly within four minutes and later withdrew a separate projection. Since it did not have an obvious polarity, it was difficult to score.

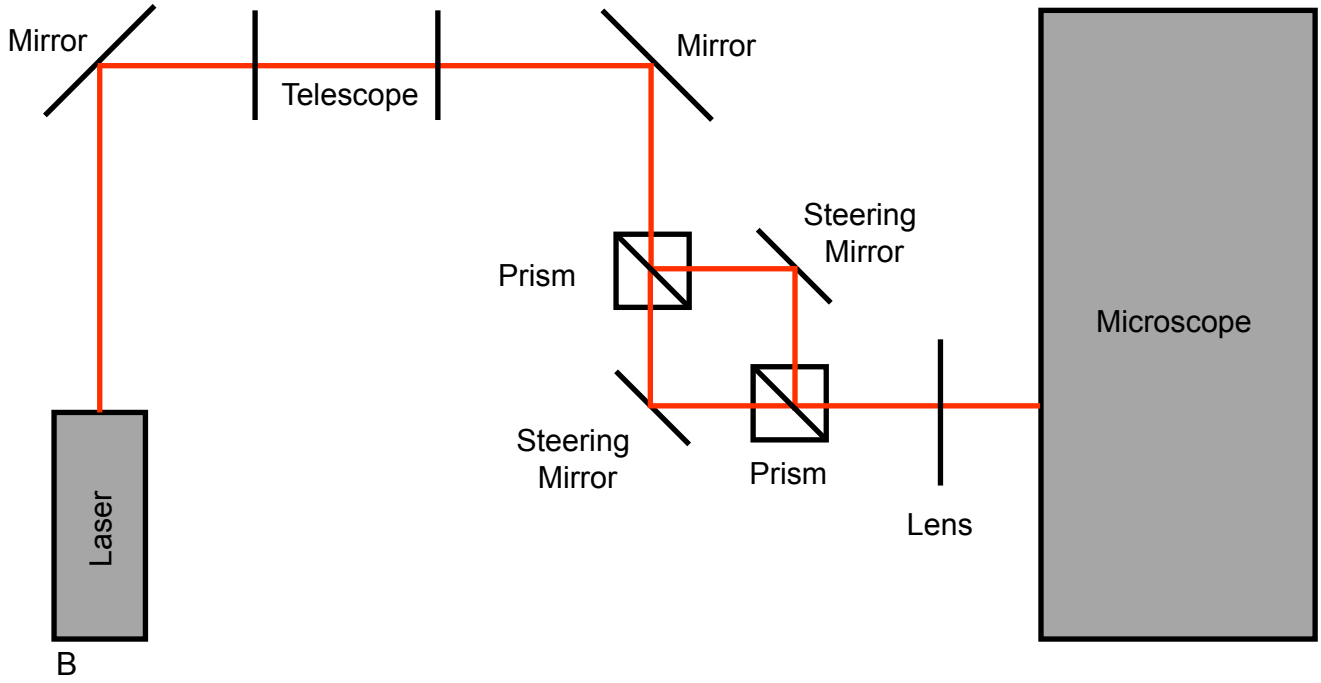
However, since it did appear to retract its projections this was characterized as a mild response to the bead and was scored a 1 by AJE and a 0 by DK.

Figure 11: This cell was contacted with a Protein-G bead on its leading edge and then it independently contacted several beads on the surface. The leading edge was completely withdrawn by 8 minutes and there were several smaller retraction events associated with contact between the cell and beads on the surface. This was characterized as a strong reaction to a Protein-G bead and was scored a 2 by AJE and DK.

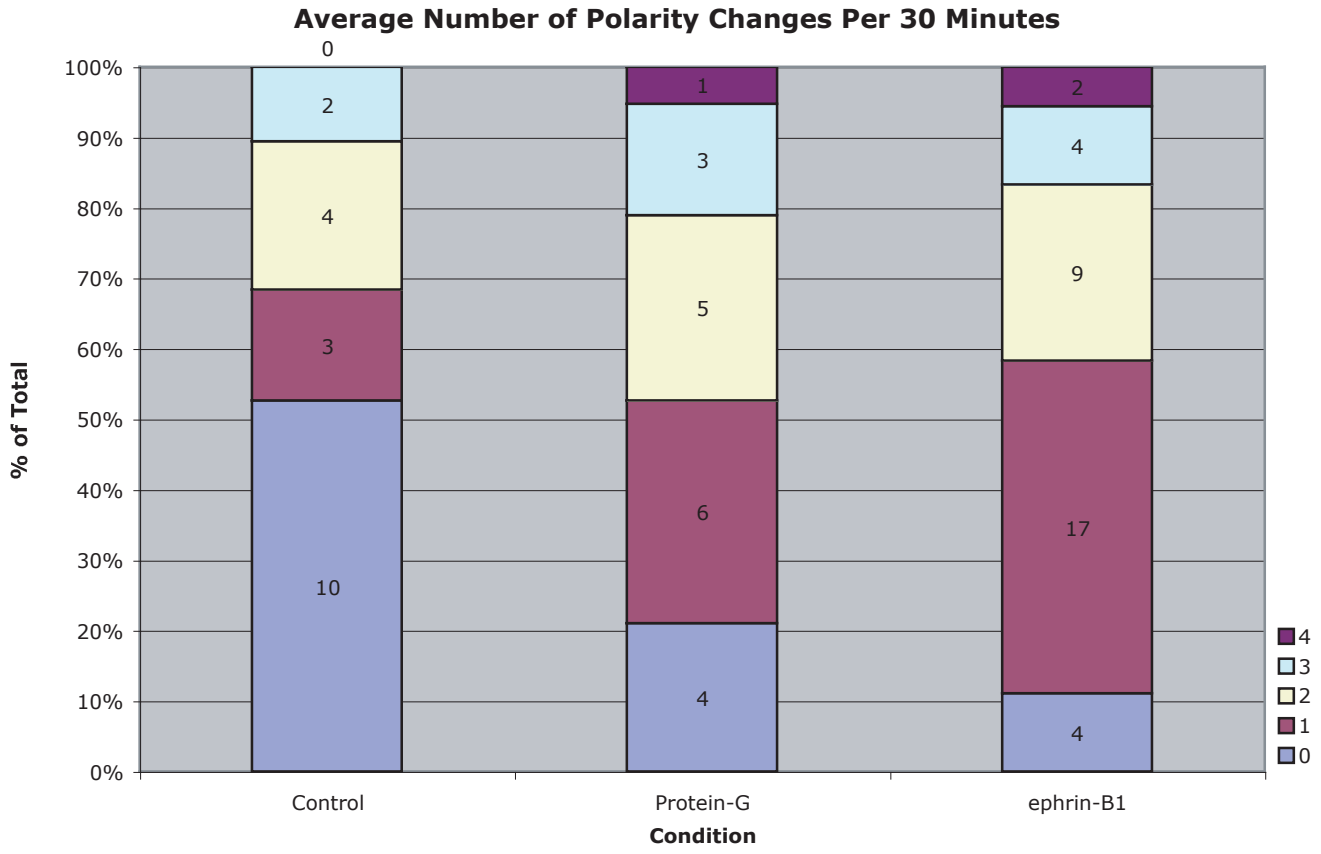
Figure 12: This cell was contacted with a Protein-G bead on its leading edge. The shift from frame 1 to 2 is a shift in the position of the stage, not a cell movement. In response to the contact this cell retracted its modest leading edge and extended in several different directions. This was scored a 1 by AJE and a 0 by DK.

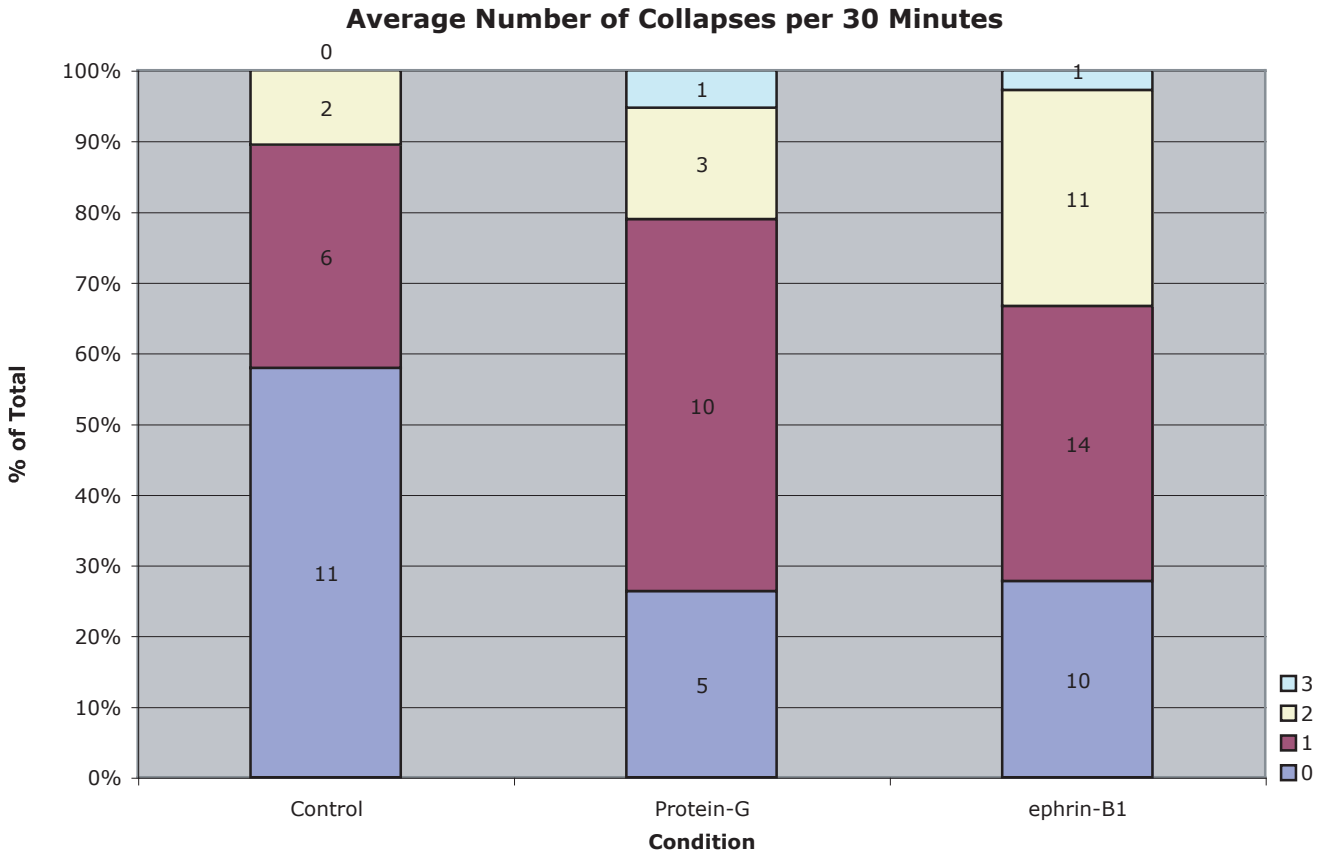


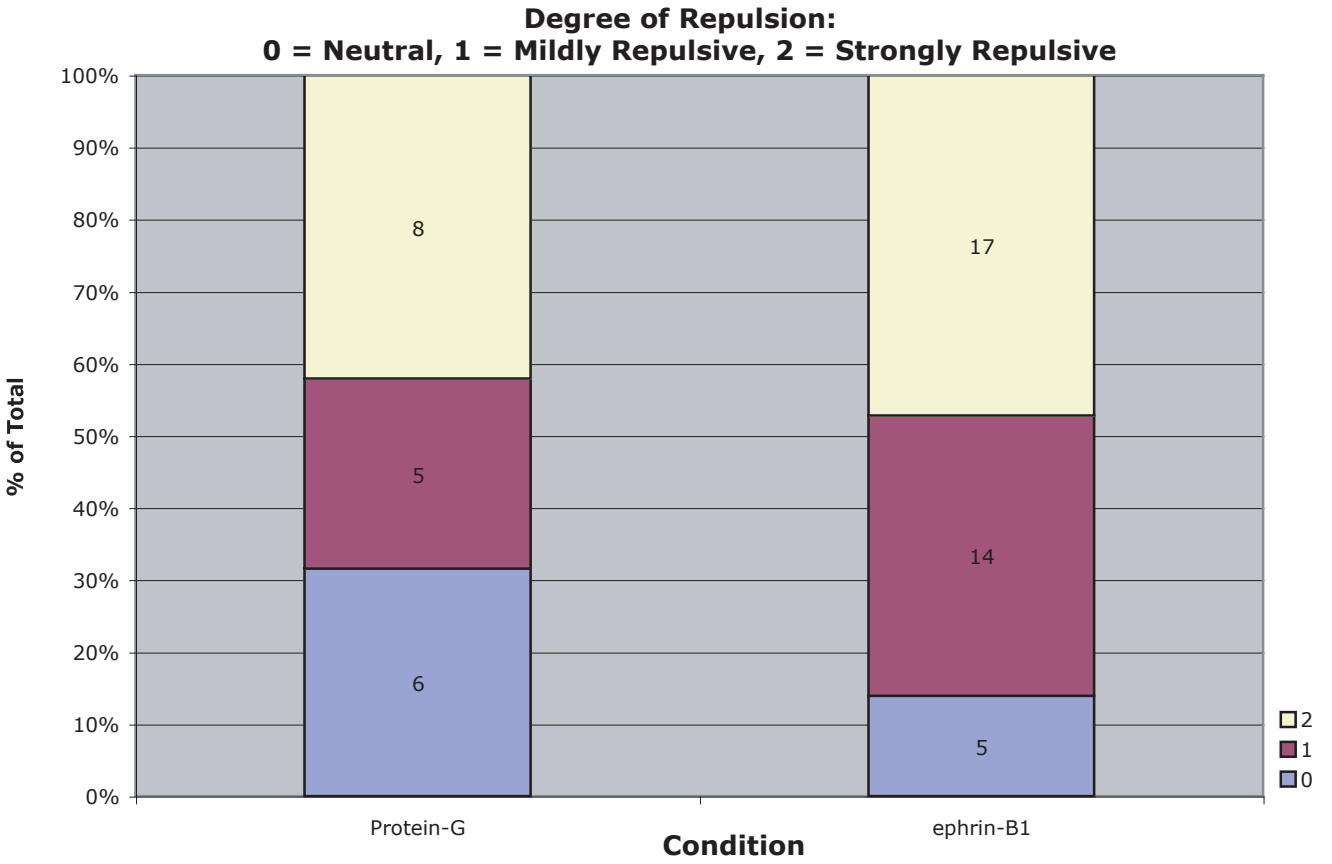
A

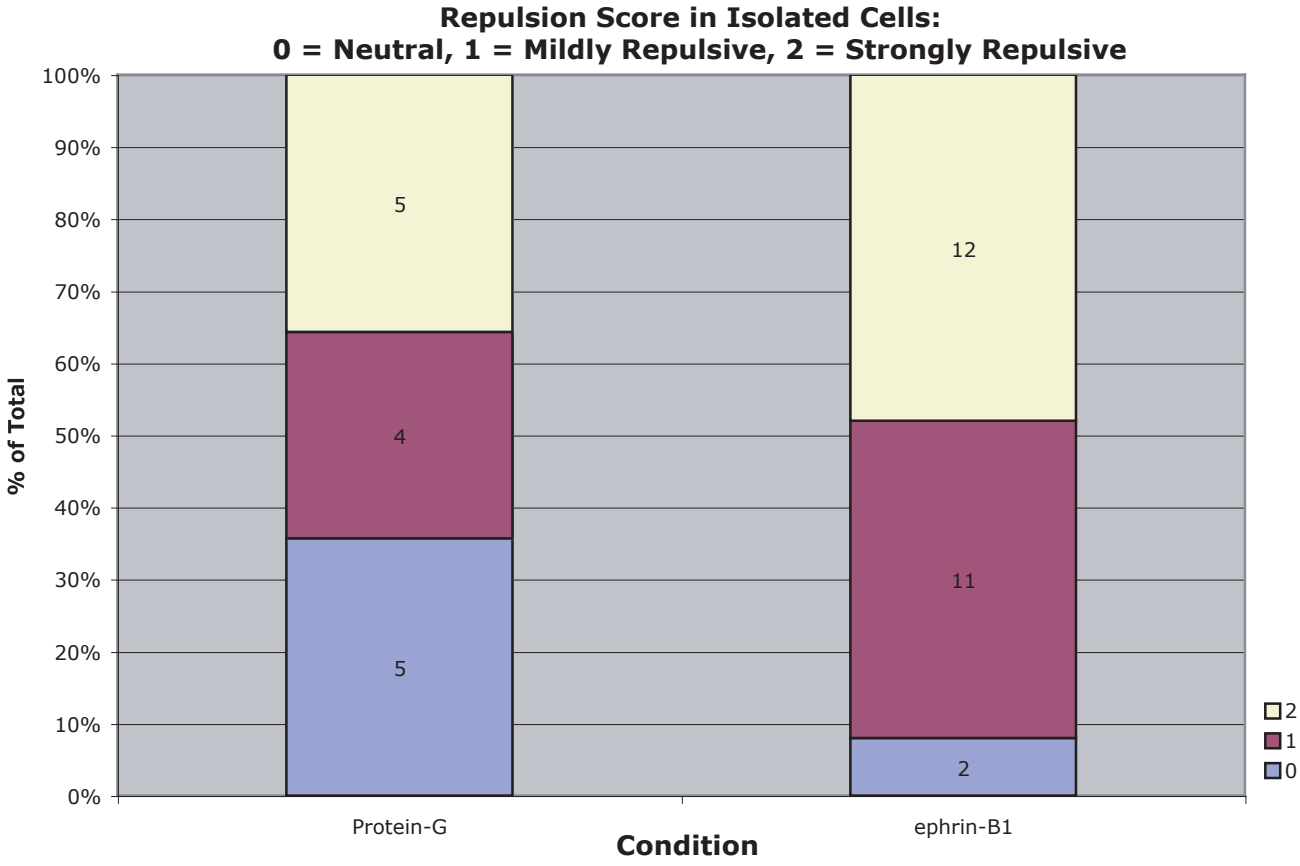

Mirror
Telescope
Mirror
Steering
Mirror
Prism
Microscope
Steering
Mirror
Prism
Lens
Laser

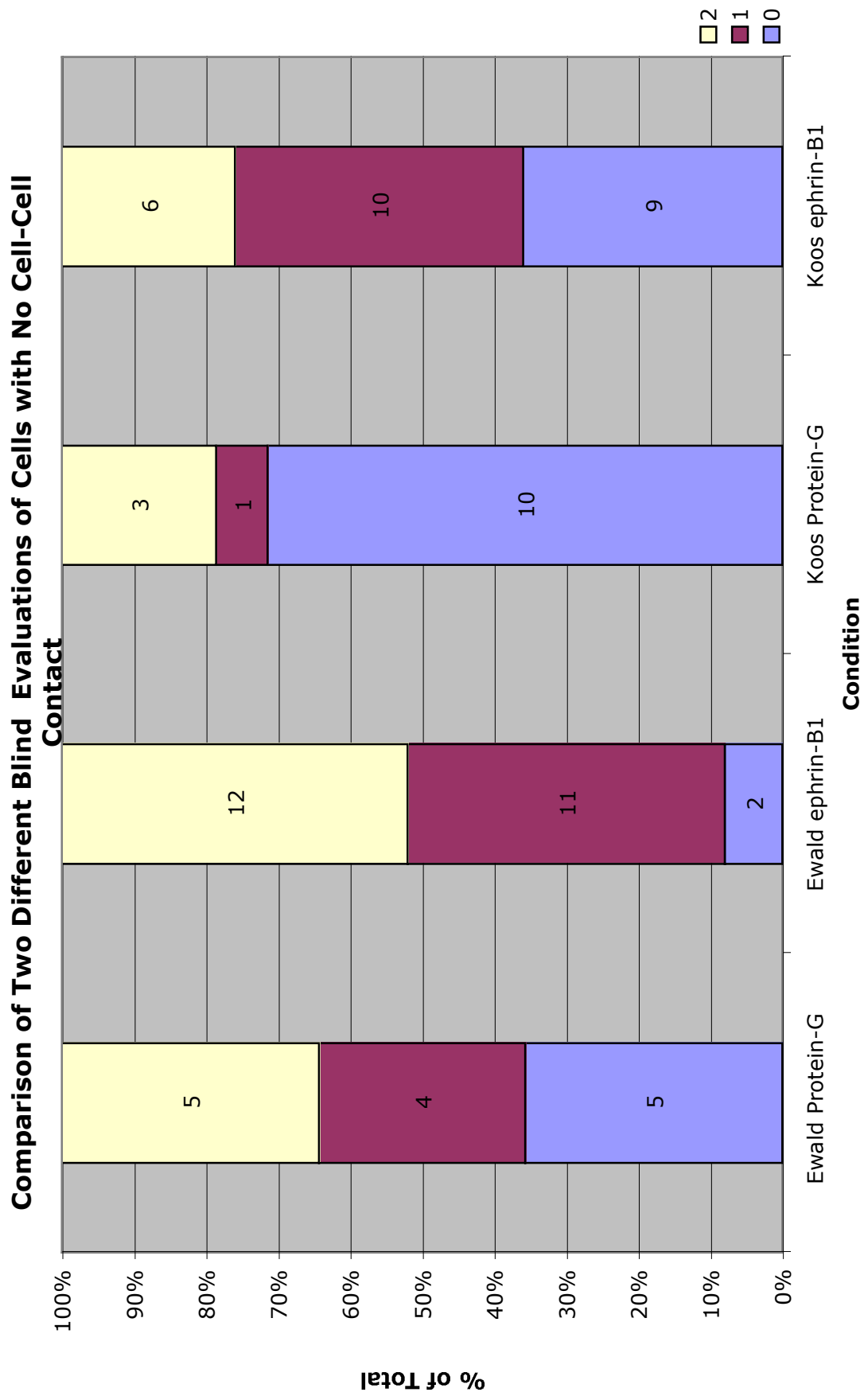
B


Average Number of Polarity Changes Per 30 Minutes
% of Total
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
2
4
3
10
1
3
5
6
4
2
4
9
17
4
Control
Protein-G
ephrin-B1
Condition
4
3
2
1
0

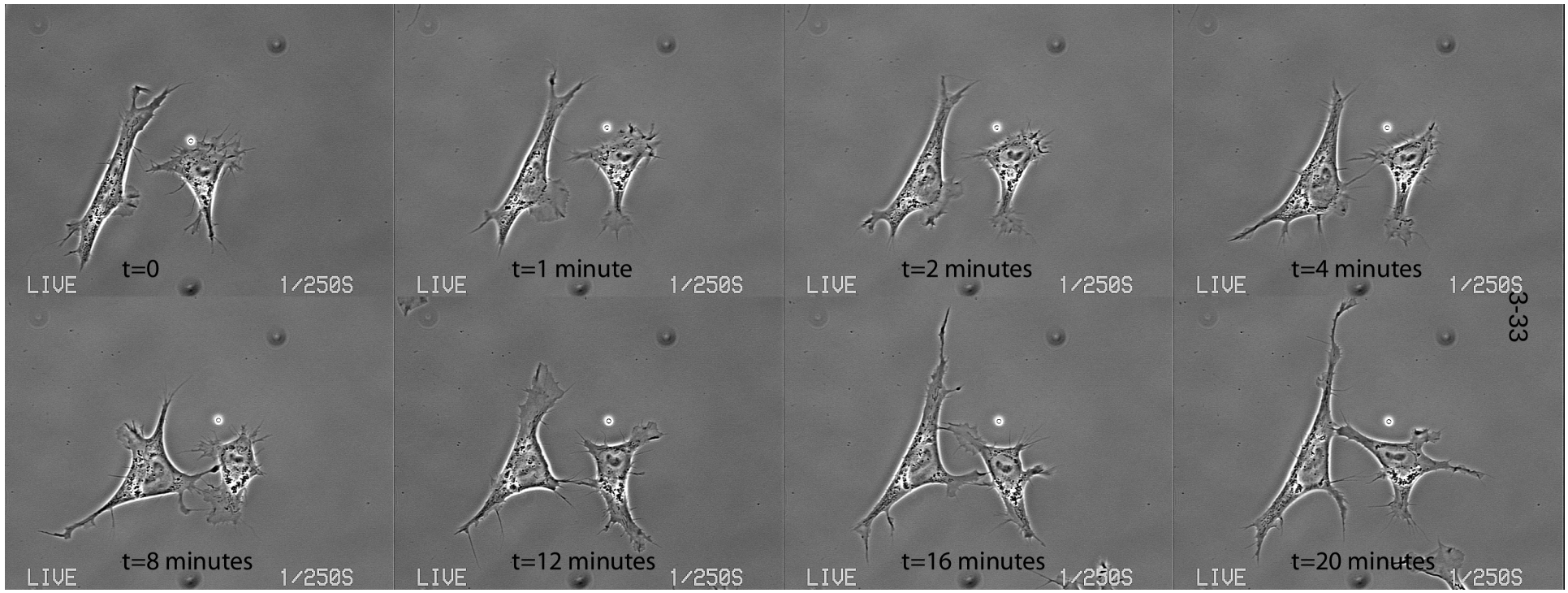

Average Number of Collapses per 30 Minutes
% of Total
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
2
6
11
1
3
10
5
1
11
14
10
Control
Protein-G
ephrin-B1
Condition
3
2
1
0


Degree of Repulsion:
0 = Neutral, 1 = Mildly Repulsive, 2 = Strongly Repulsive
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% of Total
8
5
6
17
14
5
Protein-G
ephrin-B1
Condition
2
1
0


Repulsion Score in Isolated Cells:
0 = Neutral, 1 = Mildly Repulsive, 2 = Strongly Repulsive
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% of Total
5
4
5
12
11
2
Protein-G
ephrin-B1
Condition
2
1
0


Comparison of Two Different Blind Evaluations of Cells with No Cell-Cell Contact
% of Total
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
5
4
5
12
11
2
3
1
10
6
10
9
Ewald Protein-G
Ewald ephrin-B1
Koos Protein-G
Koos ephrin-B1
Condition
2
1
0

Movie 13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LIVE
1/250S
t=1 minute
LIVE
1/250S
t=2 minutes
LIVE
1/250S
t=4 minutes
LIVE
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t=8 minutes
LIVE
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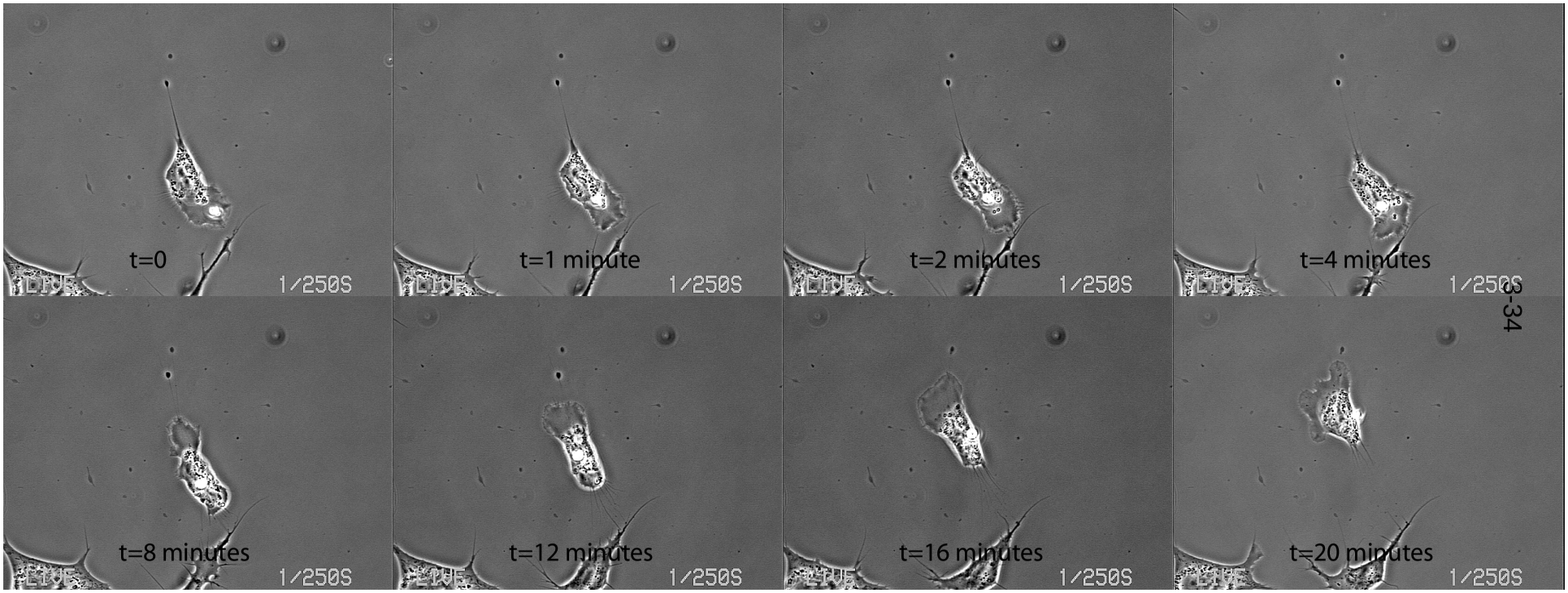
LIVE
1/250S
t=16 minutes
LIVE
1/250S
t=20 minutes
LIVE
1/250S

Movie 34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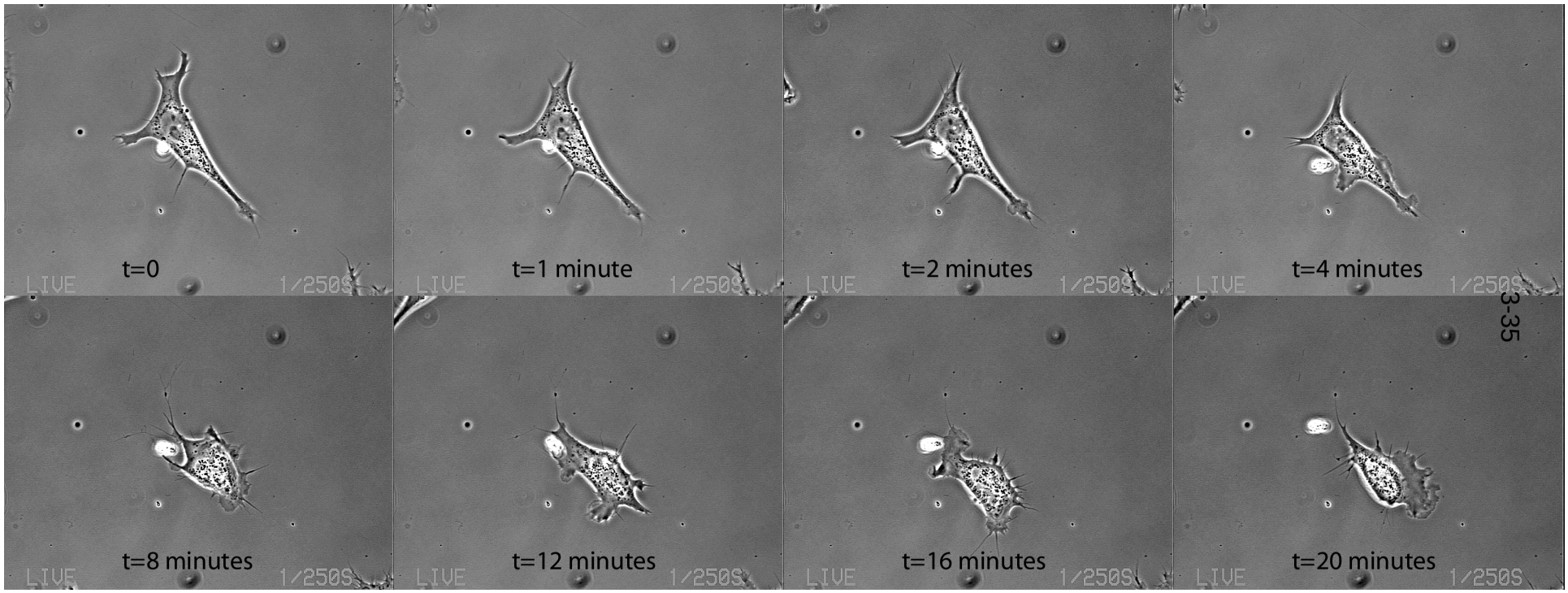
t=4 minutes
t=8 minutes
t=12 minutes
t=16 minutes
t=20 minutes
1/250S

3-34

Movie 22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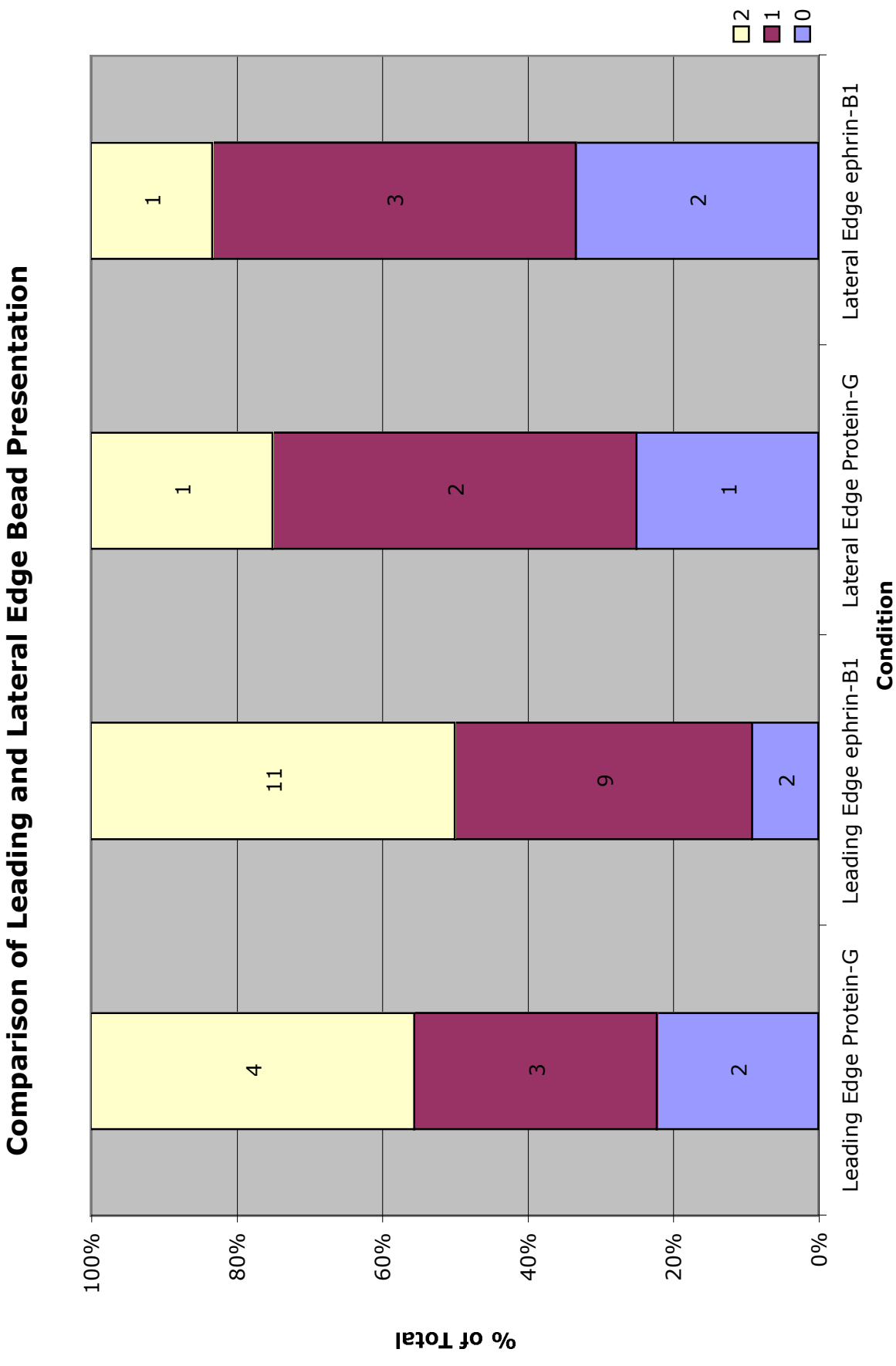
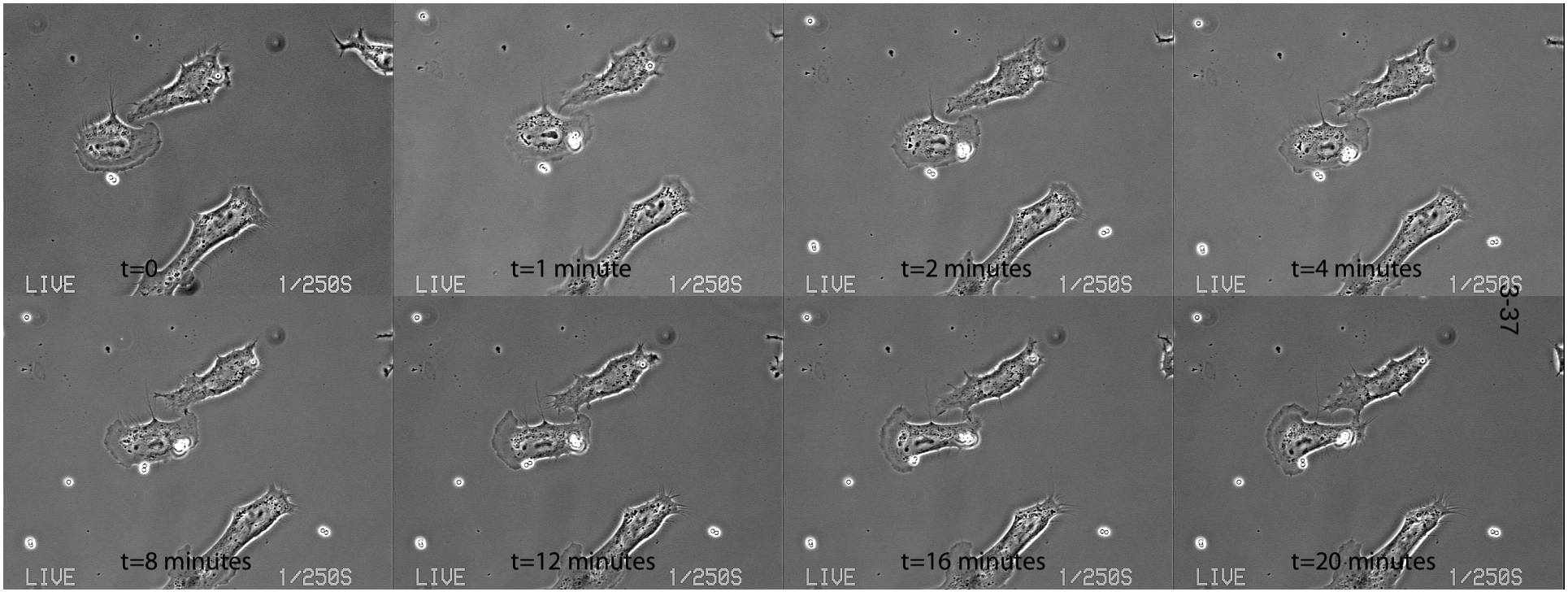
t=1 minute
t=2 minutes
t=4 minutes
t=8 minutes
t=12 minutes
t=16 minutes
t=20 minutes
LIVE
1/250S


Comparison of Leading and Lateral Edge Bead Presentation
% of Total
100%
80%
60%
40%
20%
0%
4
3
2
11
9
2
1
2
1
1
3
2
Leading Edge Protein-G
Leading Edge ephrin-B1
Lateral Edge Protein-G
Lateral Edge ephrin-B1
Condition
2
1
0

Movie 35


LIVE
t=0
1/250S
LIVE
t=1 minute
1/250S
LIVE
t=2 minutes
1/250S
LIVE
t=4 minutes
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LIVE
t=8 minutes
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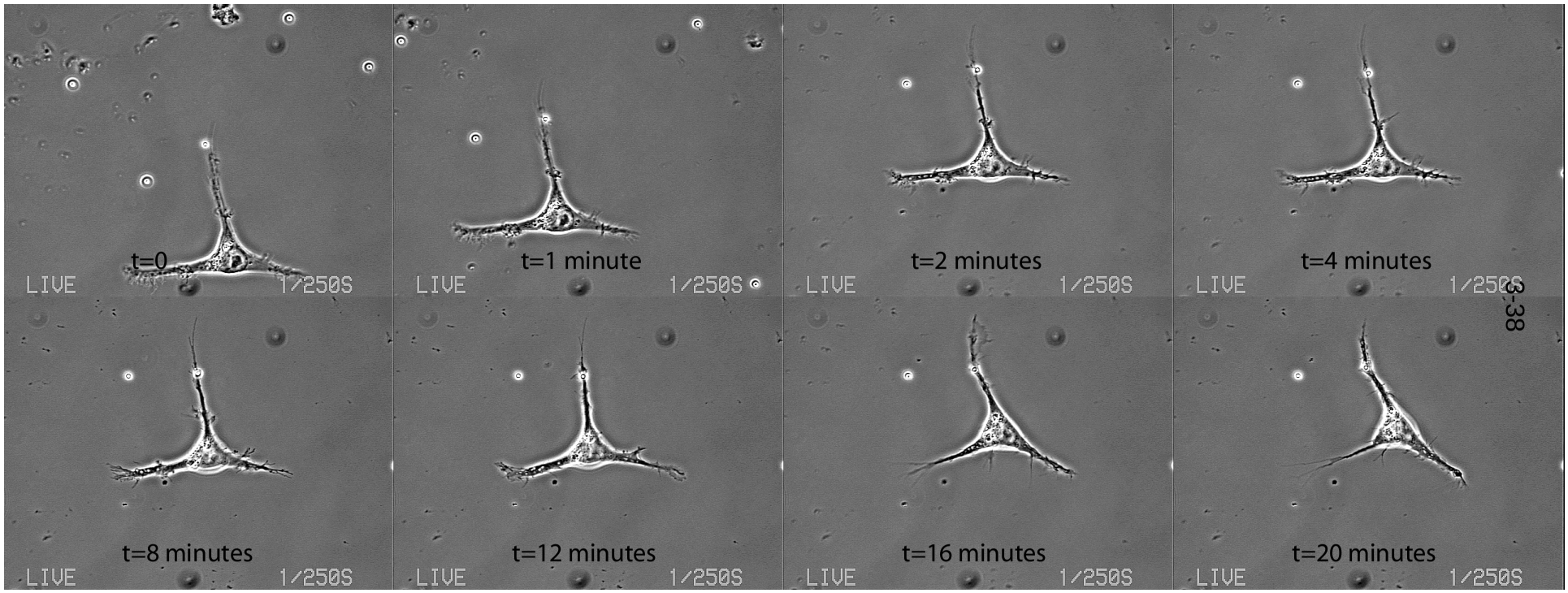
1/250S
LIVE
t=20 minutes
1/250S

Movie 5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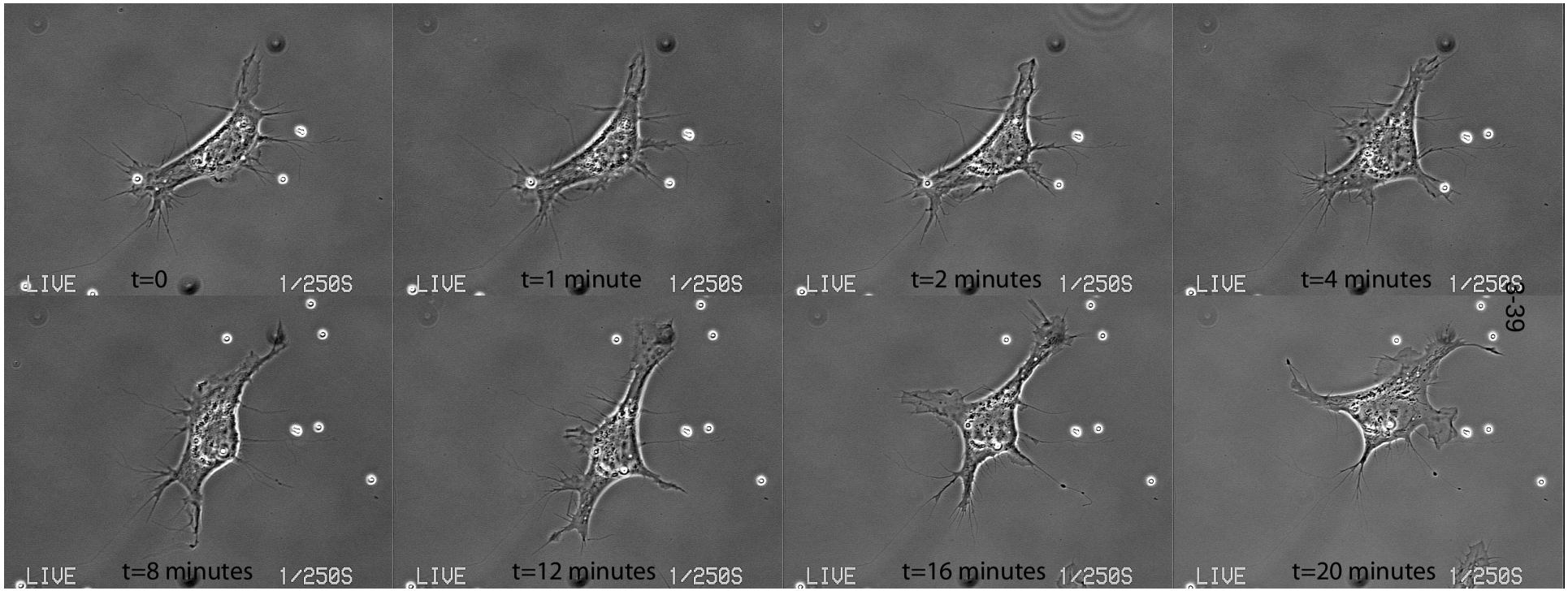
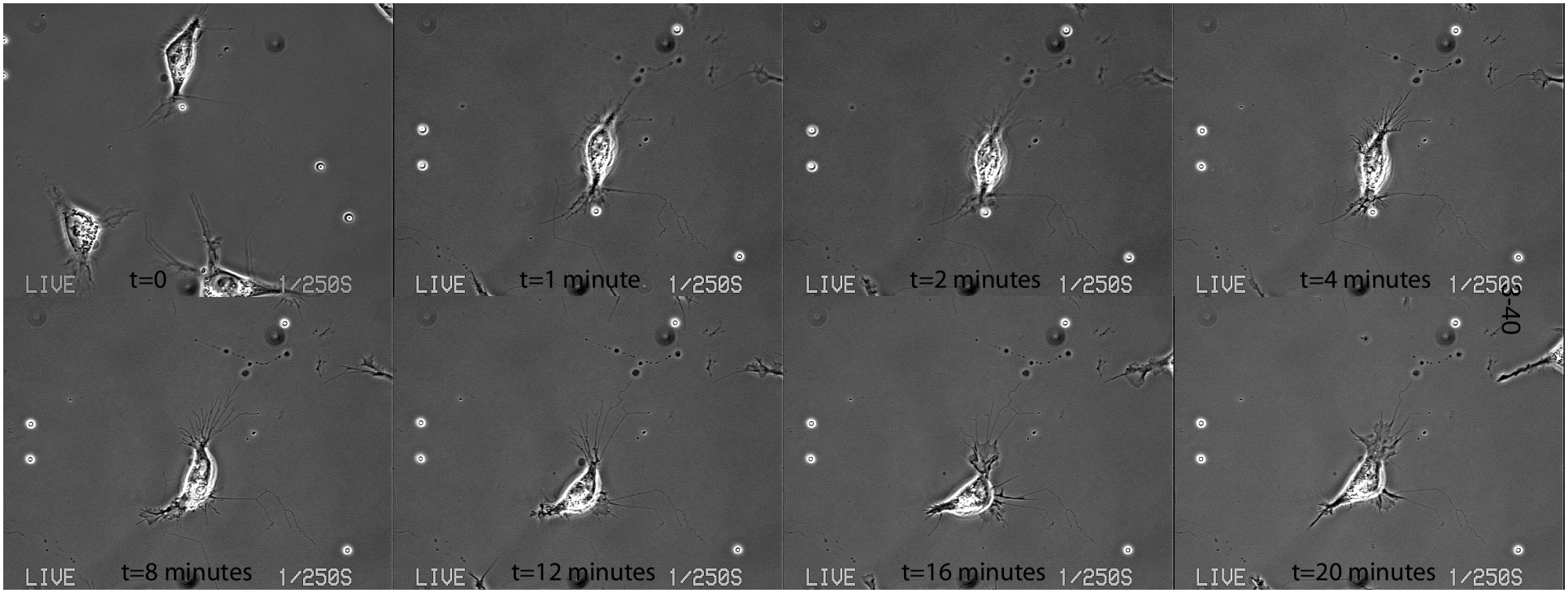
LIVE 1/250S
t=1 minute
LIVE 1/250S
t=2 minutes
LIVE 1/250S
t=4 minutes
LIVE 1/250S
t=8 minutes
LIVE 1/250S
t=12 minutes
LIVE 1/250S
t=16 minutes
LIVE 1/250S
t=20 minutes
LIVE 1/250S

Movie 16


LIVE t=0 1/250S
LIVE t=1 minute 1/250S
LIVE t=2 minutes 1/250S
LIVE t=4 minutes 1/250S
LIVE t=8 minutes 1/250S
LIVE t=12 minutes 1/250S
LIVE t=16 minutes 1/250S
LIVE t=20 minutes 1/250S

Movie 8


LIVE t=0 1/250S
LIVE t=1 minute 1/250S
LIVE t=2 minutes 1/250S
LIVE t=4 minutes 1/250S
LIVE t=8 minutes 1/250S
LIVE t=12 minutes 1/250S
LIVE t=16 minutes 1/250S
LIVE t=20 minutes 1/250S
3-40