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Utilizing phospho-histone H3 labeling in the *Drosophila* larval central nervous system to generate parametrically testable mitotic index data sets.

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Introduction

In *Drosophila*, the central nervous system (CNS) of the third instar wandering larvae continues to be a highly utilized tissue for cytological studies investigating the mitotic phenotypes of mutations thought to impact cell-cycle progression and/or chromosome dynamics. The larval CNS is unique in that, unlike most other larval organs, it persists into the adult stage (Truman, 1990), and it is comprised of two major cell types all undergoing canonical cell cycles: the neuroblasts and the ganglion mother cells (Hofbauer and Campos-Ortega, 1990). There are many published protocols available in the literature for squashing and labeling larval CNS tissue for the generation of mitotic indices in an effort to characterize mitotic defects (Gatti, 1974; Gatti and Goldberg, 1991; Bently, 2001; Williams, 1992; Bolkan, 2007; Ayeni, 2013). However, in our experience many previously described methods, while useful for analysis of chromosome morphology, did not generate data sets that were amendable to parametric statistical testing such as t-tests and ANOVAs.

In the following report, we describe a variation of our existing protocol (Apgar, 2010) for examining the progression of the canonical cell cycle of the larval CNS that utilizes the sensitivity of phospho-histone H3 (PH3) labeling to generate mitotic indices. The added sensitivity of the PH3 labeling method allows for the generation of data that satisfies the assumptions of parametric statistical testing. With these assumptions satisfied, statistical tests, such as t-test and ANOVAs, can now legitimately be used to compare the effects of different mutant alleles on cell-cycle progression.

Materials and Methods

Drosophila Stocks

The w^{1118} line was obtained from the Bloomington Stock Center (Flybase ID: FBst0006326). Flies were maintained at 25°C on *Drosophila* Diet Medium K12 (US Biological Cat #D9600-07B).

Tissue Acquisition

Wandering third instar larvae were collected and placed in a 16 well dissecting dish containing 100 µl of 1× PBS (140 mM NaCl, 2.7 mM KCl, 1.4 mM Na₂HPO₄, 1.8 mM KH₂PO₄).

The CNS was isolated using No.5 tweezers (Electron Microscopy Sciences, Hatfield, PA). All imaginal discs were removed from the CNS tissue following dissection.

Swelling and Fixing Tissue

After dissecting the central nervous system and removing the attached imaginal discs, the tissue was transferred into a new well containing 100 μ l of hypotonic solution (0.5% sodium citrate) and allowed to incubate for 10 minutes at room temperature. After 10 minutes, the tissue was moved into another well, containing 100 μ l of 4% formaldehyde in dH₂O, for 30 minutes at room temperature.

Squashing and PH3 labeling

Day One: Once fixed, the tissue was transferred to a clean microscope slide, along with 10 μ l of fixative. Next, a siliconized coverslip was carefully placed on top of the sample followed by a piece of filter paper and another microscope slide. Using a 4" vise, the sandwiched tissue was squashed for 2 minutes. Following the squash, the slide containing the tissue was lowered into liquid nitrogen for one minute. Once the slide was removed from the liquid nitrogen, the siliconized coverslip was removed with a razor blade. The sample was then incubated in 75 μ l of 1 \times PBS for 6 minutes. After 6 minutes, the 1 \times PBS solution was removed by blotting around the squashed sample with a Kimwipe[®]. The sample was then air-dried for 1 minute and placed in 75 μ l of 0.5% Triton-X in 1 \times PBS for 15 minutes at room temperature. Following the 15 minute incubation, excess Triton-X was removed with a Kimwipe[®], and the sample was then incubated with 75 μ l 3% bovine serum albumin (BSA) in membrane wash buffer (10 \times PBS, 2% Tween-20) for 30 minutes at room temperature. After 30 minutes, the BSA was removed and the slide was washed with 1 \times PBS. Using a 20cc syringe equipped with a 22 gauge blunt fill needle filled with Vaseline[®], a single line of petroleum jelly was dispensed encircling the squashed sample. Next, 75 μ l of primary antibody (rabbit α -phospho-histone H3) diluted 1:750 in 3% BSA was placed on the sample. The slides were then placed into a tupperware container lined with damp paper towels and incubated overnight at 4°C.

Day Two: Following the overnight incubation, the samples were washed by tilting the slide and gently dispensing 1 \times PBS, three times, making sure to remove the Vaseline[®] from the slide. The slide was then allowed to air dry for one minute, and subsequently incubated with 100 μ l of secondary antibody (anti-rabbit 468) diluted 1:500 in 3% BSA in the dark for 1 hour. After 1 hour, the slides were washed three times with 1 \times PBS and treated with fresh 3 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) solution for 8 minutes. After the DAPI staining the slides were then washed three more times with 1 \times PBS.

Tissue Mounting

30 μ l of Vectashield[®] Mounting Medium (Cat. No. H-1000, Vector Laboratories, Burlingame, CA) was dispensed on the squashed samples and coverslips (Fisherfinest[®], 22 \times 50-1, Cat. No. 12-548-5E) were carefully placed on the tissue. With the coverslip on the slide, the corners of the coverslip were gently tapped to ensure the entire area under the coverslip was occupied by Vectashield[®]. Nail polish was then used to seal the coverslip to the microscope slide.

Imaging the Squashed Sample

Slides were imaged using 63 \times magnification on the Zeiss LSM700 Confocal equipped with Zeiss's ZEN Black software package. Ten random, highly populated fields of view were imaged

using the 405nm and 615nm wavelength filter for DAPI and PH3 visualization, respectively. Images were saved as .tiff files for analysis using Adobe® Photoshop® elements CS5.1.

Calculating a Mitotic Index

The first step to determining the mitotic index was to count the total number of nuclei in Photoshop® CS5.1 using the count tool. Following this, the numbers of actively dividing mitotic cells (seen by PH3 labeling) were counted. The mitotic index was determined by dividing the number of actively dividing cells by the total number of nuclei. A mitotic index was calculated for each of the ten images. Calculation of the mitotic index for DAPI stained images (without PH3 labeling) was performed as described in Apgar (2010).

Statistical evaluation of distributions

Mitotic indices were calculated as described above using Excel®2010, and the square root transformations were conducted in Excel® 2010 by simply taking the square root of each mitotic index value. Mitotic indices were organized by slide ID so that comparisons could be made for both detection methods on a slide by slide basis. Datasets were imported into JMP®10 software package to visualize the distributions using the analyze distribution function. JMP®10 was also used to conduct Levene's test of variance by utilizing the Fit Y by X function using the slide ID as the grouping variable, and selecting "unequal variances". Paired t-tests were also carried out in JMP®10 software using the matched pairs utility. Data sets requiring comparisons were imported into R statistical software to conduct Kolmogorov-Smirnov Goodness of Fit analyses using standard practices.

Results

PH3 labeling provides increased sensitivity and reduces subjectivity from nuclei counts

In order to compare the labeling efficiency and sensitivity of DAPI staining versus PH3 labeling in the central nervous system (CNS) of third instar wandering larvae, ten wild type brains were dissected, squashed, and dual labeled as mentioned in the materials and methods section. From each of those slides, ten random, monolayered, and highly populated fields of view were acquired at 63× using a Zeis LSM 700 Confocal microscope equipped with Zen Black imaging software (representative images shown in Figure 1 A and B). The 100 images were then exported and counted twice using the count feature in Photoshop® CS5.1. One set of counts utilized only the DAPI signal to detect mitotically dividing nuclei and the total number of nuclei per field of view, and the other set used both the DAPI and PH3 signals to record the number of PH3⁺ nuclei and the total number of nuclei per field of view. A mitotic index was then calculated and recorded for each image, using each labeling method, by dividing the number of mitotically dividing nuclei by the total number of nuclei present in that field of view. The resulting distributions (Figure 1 C and D) show that there is a much higher abundance of zeros when using the DAPI detection method than with the PH3 method. Furthermore, the PH3 labeling method also reduced the individual subjectivity when counting mitotic nuclei such that multiple people could count the same image and reliably obtain the same number of mitotic cells, but this was not the case with the DAPI method of detection, which leaves the counting of some mitotic figures open to individual interpretation.

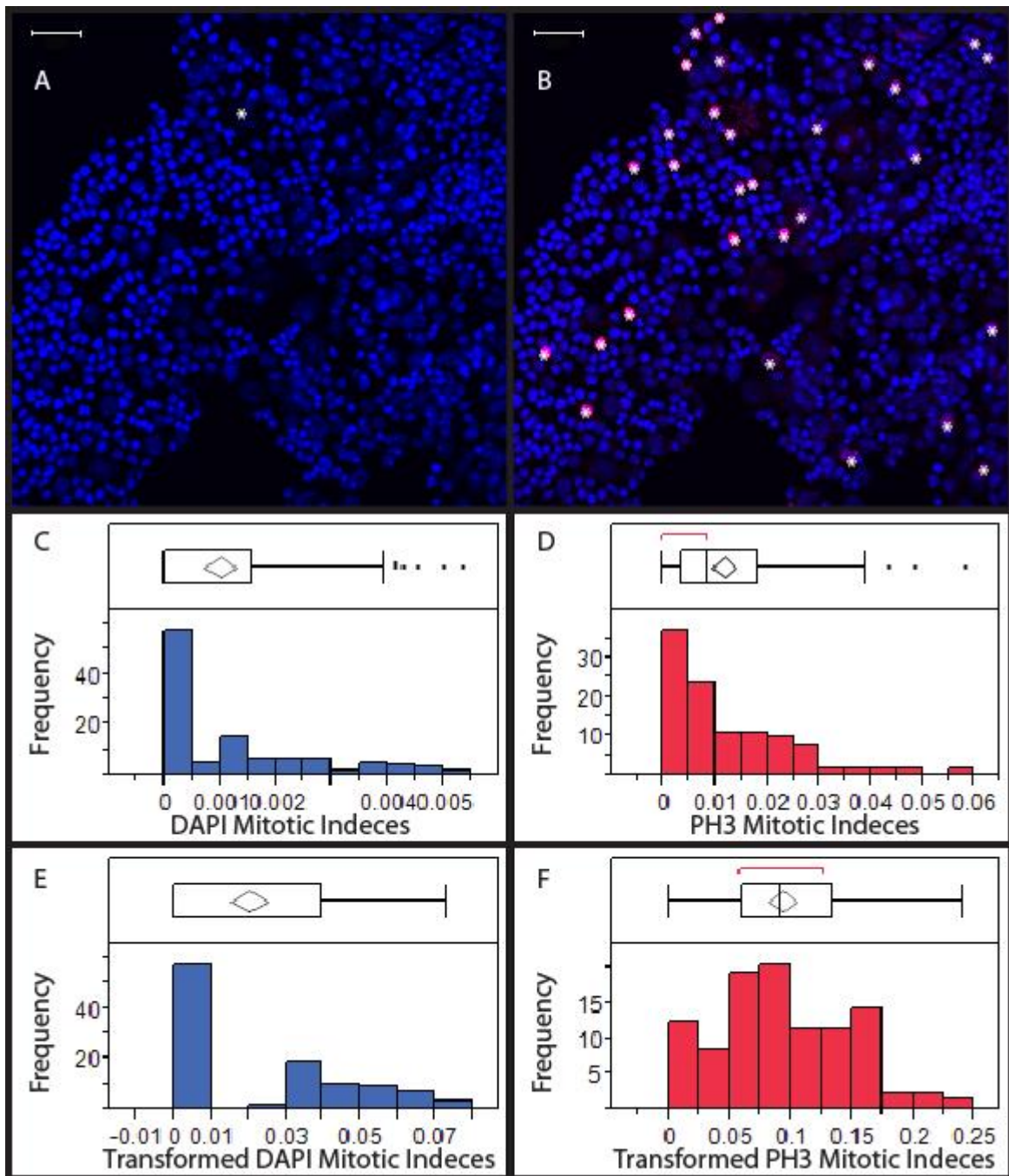


Figure 1. Increased sensitivity of PH3 labeling and its ability to generate “approximately normal” data. A: 63 \times confocal micrograph of DAPI stained nuclei depicting one unambiguous mitotic nucleus marked with an asterisk. (scale bar = 20 μ m); B: 63 \times confocal micrograph of the same slide shown in A with the PH3 signal represented. The asterisks denote mitotic nuclei that would not have been counted using only DAPI alone. (scale bar = 20 μ m); C and D: Visual representations of the distributions of mitotic indices generated using DAPI (C) or PH3 and DAPI combined (D); E and F: Visual representations of the distributions of mitotic Indices using DAPI (E) or PH3 and DAPI (F) once transformed using a square root function.

Statistical evaluation of the DAPI and PH3 mitotic index distributions

To confidently state that the PH3 detection method provides an advantage over the DAPI detection method, the distributions needed to be statistically compared to each other. To begin this comparison, a Kolmogorov-Smirnov Goodness of Fit analysis was conducted to compare the DAPI and PH3 distributions. This test validated that the two distributions were significantly different ($P < 2.2e^{-16}$). Though the PH3 labeling method generates a significantly different distribution, which contains less zeros and reduces ambiguity in counting mitotic figures, observation of the resulting DAPI and PH3 distributions (Figure 1 C and D, respectively) easily show that neither of these distributions appears to be normally distributed. Furthermore, significant results from Levene's test for homogeneity of variance for both DAPI ($P < 0.0001$) and PH3 ($P = 0.0264$) distributions demonstrate that both suffer from heterogeneous variance. Thus, the skewed nature of the distributions and homogenous variance for these data sets would violate two of the three assumptions that must be met to reliably conduct a parametric test.

The DAPI and PH3 distributions were transformed using a basic square root transformation and then reevaluated for the ability to meet the assumptions of normality and homogenous variance. The variances of the transformed distributions were tested again using Levene's test and resulted in a significant result for the transformed DAPI data set ($P = 0.0001$) but not for the transformed PH3 data set ($P = 0.7393$). Therefore the transformed PH3 distribution now meets the assumption of homogeneous variance; furthermore, the square root transformation also makes the PH3 distribution, but not the DAPI distribution, appear much more normally distributed (Figure 1 E and F). However, a significant result from a Kolmogorov-Smirnov Goodness of Fit analysis comparing the transformed PH3 distribution to a generated normal distribution ($P < 2.2e^{-16}$) suggests that the data are "approximately normal" but not completely normally distributed. Finally a paired t-test that was conducted, using slide numbers for grouping, evaluating both transformed distributions and a P-value < 0.0001 indicates that the transformed PH3 data generate a different mean both among and within the groups when compared to the transformed DAPI data set.

Discussion

The use of mitotic indices as means of assessing cell cycle progression, and even as a diagnostic test for the presence of cancerous cells, has been utilized in many model systems from cell culture to canines, displaying the versatility and potential sensitivity of this type of assay (Romansik, 2007; Baak, 2009). In the *Drosophila* community the larval CNS has been used extensively to evaluate mutants for impacts on cell cycle progression and chromatin morphology, and as such there are many different approaches for utilizing these tissues in the literature (Gatti, 1974; Gatti and Goldberg, 1991; Bently, 2001; Williams, 1992; Bolkan, 2007; Ayeni, 2013). The popularity of this specific tissue for this type of analysis can be explained by the ease of dissection of the tissue, and the fact that the CNS is comprised of two major cell types all undergoing canonical cell cycles: the neuroblasts and the ganglion mother cells. The neuroblasts can divide either symmetrically or asymmetrically resulting in two neuroblasts or a neuroblast and a ganglion mother cell, respectively. Ganglion mother cells can then divide only once, producing two daughter cells that differentiate into neurons (Hofbauer, 1990; Truman, 1990). Due to the proliferative nature of these cells, the larval CNS contains proliferation centers that produce characteristic patterns of cell cycle progression in specific regions (Truman 1988; Truman, 1990; Ito, 1991). These stereotypical patterns of cell cycle progression can be visualized using thymidine analogue incorporation assays in the brain (Apger, 2010). For this reason our lab attempts to reduce bias and inflated counts of mitotic nuclei by acquiring 10 random highly populated fields of view from each larval CNS that is analyzed as

opposed to counting single fields of view, acquiring adjacent fields of view, or attempting to count entire brains (Bolkan, 2007; Baak, 2009; Apger, 2010).

As stated previously there are many established protocols in the literature that can be used to visualize mitotic cells in the larval CNS; however, the methods used to calculate mitotic indices vary greatly from one source to another (Gatti, 1974; Gatti and Goldberg, 1991; Bently, 2001; Williams, 1992; Bolkan, 2007; Ayeni, 2013). Furthermore, the statistical methods used to compare these mitotic indices are often not clearly reported, or commonly employ non-parametric testing to elucidate non-quantitative differences between treatments (Gatti, 1974; Gatti and Baker, 1989; Gatti and Goldberg, 1991; Bently, 2001; Williams, 1992; Bolkan, 2007; Ayeni, 2013). Our lab has historically followed the protocol listed in Apger (2010) which is roughly based on the protocols described by Gatti and Goldberg (1991) with some modifications. However, in our experience with this protocol, we commonly acquire data that do not meet the assumptions required for parametric testing (*i.e.*, normality, homogenous variance, and independence of samples/error), thus we must rely upon non-parametric tests for comparisons (Glass, 1972; Zimmerman, 1998). The assumptions that we usually failed to satisfy were those of normality and homogeneous variance, and these issues resulted from an abundance of zeros in our data sets acquired using DAPI staining (see Figure 1 C and E). The approach utilized to solve these issues was to increase our assays sensitivity such that the number of mitotic indices with a value of zero would be reduced, but maintain the same sample size. The added sensitivity of PH3 antibodies to detect mitotically dividing cells throughout M phase is prevalent in the literature and has been conducted in the larval CNS (Hans, 2001; Bolkan, 2007; Pennetier, 2012). Therefore, we decided to utilize the added sensitivity of the PH3 antibody labeling approach with our currently established protocol from Apger (2010).

A comparison of the distributions obtained by the DAPI labeling versus the PH3 labeling strategy (Figure 1 C and D) clearly demonstrated that the added sensitivity of PH3 antibody labeling did reduce the overall number of mitotic indices with a value of zero. However, the data obtained by both methods remained heavily skewed and suffered from heterogenous variance. The reduction in zero values provided by the PH3 labeling approach, but not the DAPI staining approach, allowed for a standard square root transformation to make the PH3 distribution both “approximately normal” and contain homogenous variance. Given that our PH3 distribution now meets the assumptions of homogenous variance and independence of samples/ errors, we can legitimately conduct parametric analyses with “approximately normal” distributions, because the slight violation of normality does not have as much of an impact on parametric tests as violations of the assumptions that our data satisfies (Glass, 1972). Furthermore, reports in the literature state that parametric tests remain more robust than non-parametric tests when using roughly normal data (Zimmerman, 1998).

In summary, we have demonstrated that the use of PH3 antibody to label mitotic cells using the method outlined in Apger (2010) will result in a distribution that is amendable to parametric testing after a standard square root transformation is applied to the data. This will allow for a variety of parametric tests to be conducted on the resulting data in a way that will provide quantitative differences between mutants/treatments.

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Protocol: Utilizing phospho-histone H3 labeling in the *Drosophila* larval central nervous system to generate parametrically testable mitotic index data sets

Reagents

1× PBS (140 mM NaCl, 2.7 mM KCl, 1.4 mM Na₂HPO₄, 1.8 mM KH₂PO₄)
0.5% Triton-X in 1× PBS
3% BSA in membrane wash buffer (10× PBS, 2% Tween-20)
0.5% sodium citrate in dH₂O
4% formaldehyde in dH₂O
Sigmacoat[®] (Cat. No. SL-2, Sigma-Aldrich Co. LLC, St. Louis, MO)
3 µg/ml DAPI (4',6-diamidino-2-phenylindole) in 1× PBS
Primary Antibody (rabbit α-phospho-histone H3)
Secondary Antibody (anti-rabbit 468)

Equipment

Slides (Fisherbrand[®] 25 × 75 × 1.0 mm, Cat. No. 22-034-486)
Vectashield[®] Mounting Medium (Cat. No. H-1000, Vector Laboratories, Burlingame, CA)
Coverslip (Fisherfinest[®], 22 × 50-1, Cat. No. 12-548-5E)
Liquid nitrogen
No. 5 Tweezers (Electron Microscopy Sciences, Hatfield, PA)
Blotting paper
Vaseline[®]
4" Vise
Tupperware containers
Paper towels
Kimwipe[®]
Razorblade

Dissecting and labeling 3rd instar larval CNS

DAY ONE

1. Using a 16 well dissecting dish, dissect the 3rd instar wandering larval CNS in 100 µl of 1× PBS. Make sure to remove the attached eye and wing imaginal discs.

2. Transfer the tissue into a second well filled with 0.5% sodium citrate (hypotonic solution) for 10 minutes.
3. After 10 minutes, move the CNS into another well filled with 4% formaldehyde (fixative) for 30 minutes.
4. Transfer the CNS, along with 10 μ l of fixative, onto a clean slide and carefully lower a siliconized coverslip on top of the tissue.
**Helpful Hint: Make sure to mark the location of the tissue on the bottom of the slide after placing the siliconized coverslip.*
5. To create the squashed sample, a vise will be utilized. Create a microscope slide sandwich by placing a sheet of blotting paper and another blank slide on top of the sheet. Place the sandwich in the vise, tighten, and let squash for 2 minutes.
**Helpful Hint: Be careful when handling the microscope slides. Shuffling the slides can result in poor images.*
6. After 2 minutes, freeze the sample to the slide in liquid nitrogen for one minute. Once the slide has been removed from liquid nitrogen, pop off the siliconized coverslip using a razor blade.
7. Next, wash the sample with 75 μ l of 1 \times PBS for 6 minutes.
**Helpful Hint: If performing multiple dissections, you may prolong this wash period.*
8. Remove the PBS solution from the sample by tilting the slide and blotting carefully with a Kimwipe[®]. Make sure not to disrupt the sample.
9. Treat the dried slide(s) with 75 μ l of 0.5% Triton-X solution in 1 \times PBS for 15 minutes.
10. Remove excess Triton-X solution in PBS as describe above.
11. After removing the solution, incubate the sample in 75 μ l 3% BSA for 30 minutes.
12. Following the 30 minute incubation, wash the slide once with 1 \times PBS.
13. Using a syringe of Vaseline, encircle the sample. This keeps the primary antibody (see next step) on the dissected sample.
14. Place 75 μ l of primary antibody, diluted in 3% BSA, onto the sample (if using phospho-H3 perform a 1:750 dilution).
15. Next, place the sample slide(s) in a humid chamber and incubate at 4°C overnight. Create a humid chamber by lining the interior of a Tupperware container with damp paper towels.

DAY TWO

1. Gently wash the slide(s) three times with 1 \times PBS.
2. After the third wash, remove excess PBS with a Kimwipe[®].
3. Next, incubate the sample in 100 μ l secondary antibody, diluted (1:500) in 3% BSA, in the dark for 1 hour.
4. Following this incubation, wash the sample an additional three times with 1 \times PBS.
5. Again, remove excess PBS with a Kimwipe[®].
6. Continuing, place 3 μ l of DAPI in 1 \times PBS [3 μ g/ml] onto the sample(s) for 8 minutes.
7. Gently wash the slide(s) three times with 1 \times PBS and then dry by removing excess PBS with a Kimwipe[®].
8. For fluorescence, mount the sample(s) in Vectashield[®] H-1000 and gently place down a non-siliconized coverslip.
9. Seal the coverslip around the edges with nail polish to prevent Vectashield[®] from evaporating.