Introduction

When Tissue Culture was first devised by Ross Harrison in 1907 it was done so based on the explantation of whole fragments of tissue with a view to studying them as tissues in isolation. It was observed, however, that cells often grew out from these tissues in a sheet to form a monolayer on the supporting glass substrate. This divergence in growth properties, that is, growth as a migratory and potentially proliferative monolayer versus residence within the original explant, set the stage for future approaches to the culture of animal cells. Some researchers believed in the retention of histological structure and the possibility of organotypic function while others looked towards the biology of individual cells. The former required retention of cell-cell interactions, histological structure and characteristics, and enabled the study of development of tissues. The latter gave rise to propagated cell lines which laid down the basis for most of modern cellular and molecular biology.

To study complex problems of the central nervous system (CNS) such as the demyelinating disorder multiple sclerosis (MS) where there is much interplay between different cell types, we chose to utilize an organotypic system that can preserve the interactions between cells of the appropriate lineage, the appropriate stage in that lineage and in the appropriate intracellular regulatory environment. With an interest toward better understanding the formation of CNS myelin, we present here light and scanning laser confocal microscopy images of the cerebellar slice cultures (Notterpek et al., 1993). Better understanding of the formation of myelin will not only benefit MS but other disorders of myelin as well as spinal cord injury in which both nerve and myelin repair are essential for proper nerve function.

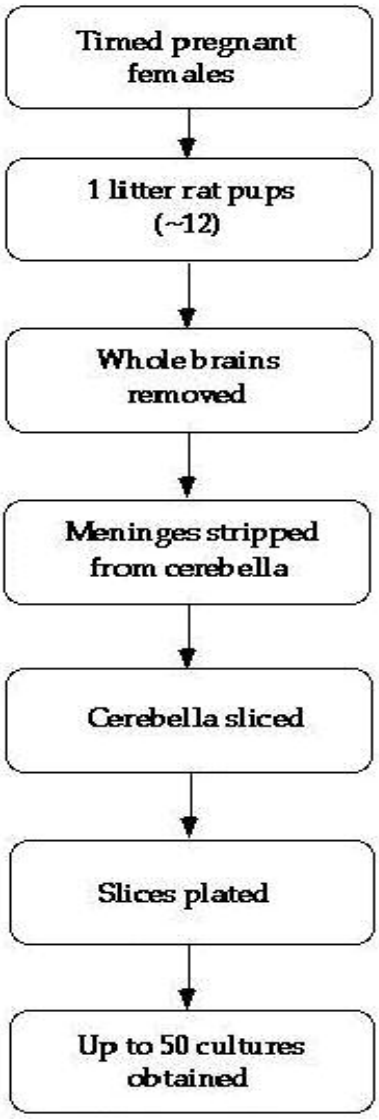
Bornstein was the first to employ the method of tissue culture to the study of myelin in 1961 (Bornstein and Appel, 1961) and an abundance of papers followed. The majority of Bornstein's work focused on studies of demyelination *in vitro* using antibodies isolated from serum of animals with experimental "allergic" encephalomyelitis (EAE), an animal model of human MS (Bornstein, 1963). These studies were thoroughly reviewed by Seil in 1977. We have coupled the organotypic cerebellar slice culture method of Notterpek et al. (1993) with light and confocal microscopy to produce visual images of the cellular sequence associated with the formation of CNS myelin.

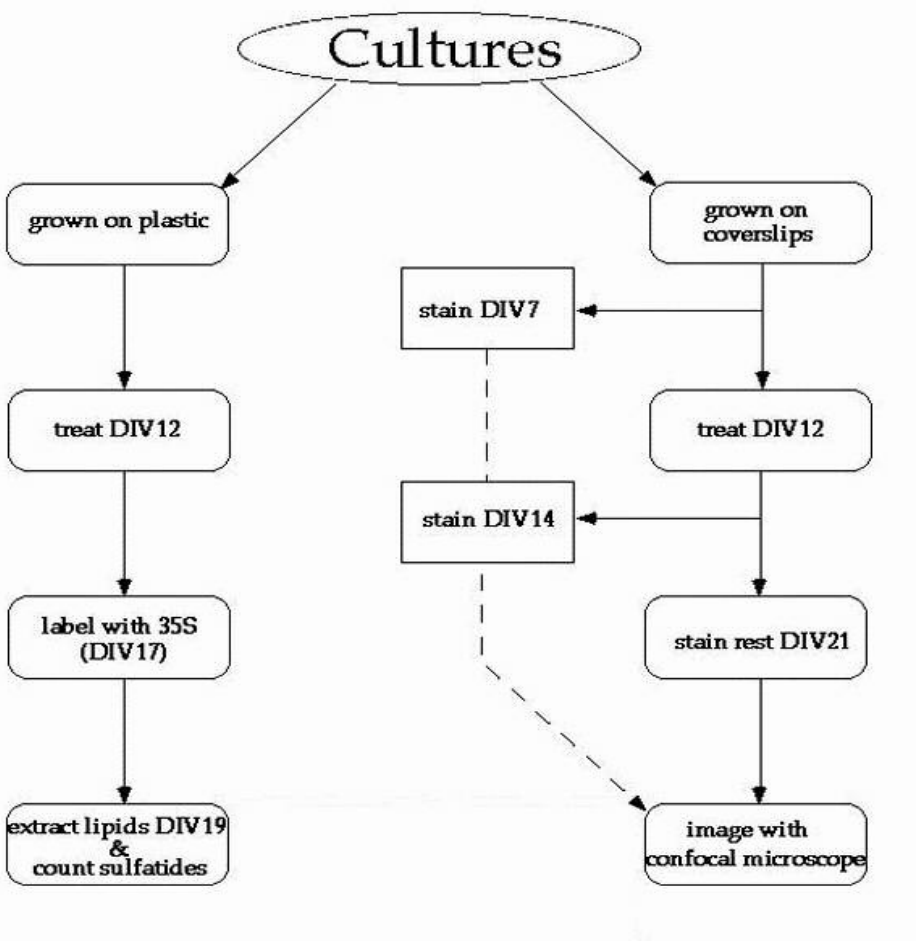
Materials and Methods

We used the exact culture method of Notterpek et al. (1993). The Experiment itself starts with timed pregnant female rats. Upon birth of their litter, whole brains from the neonatal rat pups are excised and the meninges are stripped from the cerebella with a dissecting microscope. The cerebella are dissected and chopped with a tissue microtome into 400µm thick slices of cerebellum. Over the course of 21 days *in vitro* (DIV) the cells and neurons proliferate out of the core of the slice to establish a primary culture.

The slices were placed on 6-well plates coated with poly-L-lysine and fed media for a period of 19 days (Notterpek et al., 1993). The maximum rate of myelination as determined by Notterpek et al. (1993) occurs on DIV 17. During the last two days *in vitro* the cultures were incubated in sulfur-35 labeled media, though results are not presented here. The lipids were then extracted from each culture by the method of Cardwell and Rome (1988). Sulfolipids are myelin-specific

and the number of liquid scintillation counts in each lipid fraction reflects the relative amount of myelin produced in each culture. Flow charts of the cerebellar slice culture technique follow.





Light microscopy images were obtained using an Olympus inverted microscope. Confocal fluorescence images were obtained with a scanning laser confocal microscopy unit. Image analysis was performed on a Macintosh Performa 5200CD computer using the public domain NIH Image program (available on the Internet at http://rsb.info.nih.gov/nih-image/).

We observed biochemical changes in myelin lipid content during human myelination (Husted et al., 1994) and chose antibodies that would permit observation of the morphological changes to CNS cells that occur during the course of myelin formation. Anti-Neurofilament (NF) labels specifically for axons, Anti-Glial Fibrillary Acidic Protein (GFAP) labels for astrocytes, and Anti-Galactocerebrosides (GalC) are specific for oligodendrocytes, the myelin-forming cells in

culture. The acquisition of confocal images requires attachment of primary antibodies specific to the cell of interest and secondary antibodies with fluorescent probes that are illuminated with a laser. For double and triple labeling studies, antibodies must be chosen with wavelengths sufficiently far apart to simultaneously detect different cells by switching the confocal filters. In addition, primary and secondary antibodies must be chosen from different sources so that they will bind to one another. The following Table summarizes details of the primary and secondary antibodies used to acquire confocal images of myelin formation in cerebellar slice cultures.

Cell type	**Primary antibody**	**Secondary antibody**	**Fluorophore/Protein Absorbance Ratio**
Astrocyte (blue)	Guinea pig Anti-Glial Fibrillary Acidic Protein (Advanced ImmunoChemical, Long Beach, CA)	Cy 5-conjugated AffiniPure Donkey Anti-Guinea Pig IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA)	A652/A280 = 2.40
Neuron (red)	Mouse Anti-Neurofilament M C-Terminal Polyclonal Antibody (Chemicon International, Inc., Temecula, CA)	Texas Red dye-conjugated AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA)	Texas Red sulfonyl chloride A596/A280 = 0.60
Oligodendrocyte (green)	Rabbit Anti-galactocerebroside Polyclonal antibody (Chemicon International, Inc., Temecula, CA)	Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Rabbit IgG	Fluorescein Isothiocyanate 3.4 moles FITC per mole IgG

Results

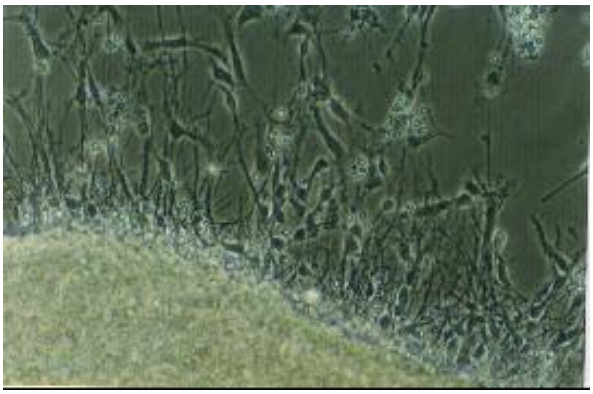
Light Microscopy images of Cerebellar Slices in Culture

The cerebellar slices were inspected daily by light microscopy to characterize developmental changes in tissue organization.

DIV 0 40X

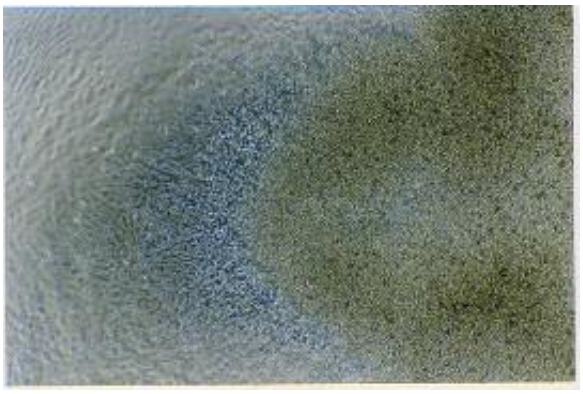
This image was captured 2 hours after plating at a magnification of 40X.

DIV 2 40X
By DIV2 the slice is becoming circular while the folds of the cerebellum are less evident. Some migration is occurring away from the core of the slice.



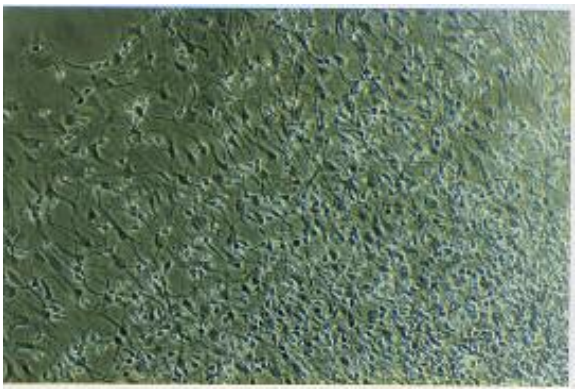
DIV 2 200X

This rapidly established culture has started to migrate out of the periphery of the tissue. At only DIV 2 most of the cells shown are mixed microglial in origin.



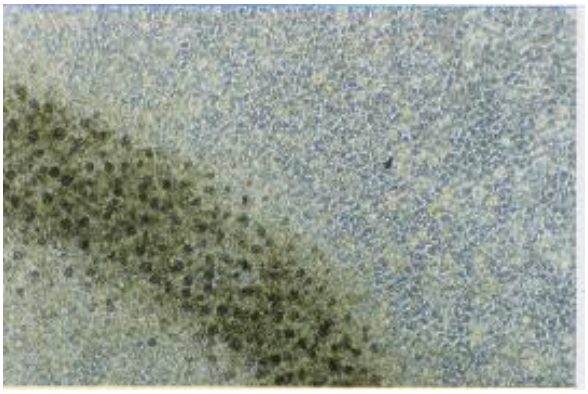
DIV 5 40X

This image captured at 40X represents some of the initial cellular spreading away from the tissue. The slice is in the right half of the image and is composed mostly of the yellowish (brighter) microglia as well as a few darker, round macrophages.



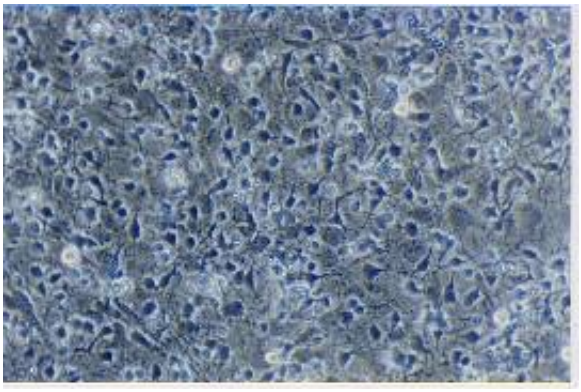
DIV 5 100X

This is the same slice imaged above, while here the focus is on the migrating cells. At 100X you are able to view the fibroblasts and microglia migrating away from the slice which would be located in the lower right-hand side of the image.



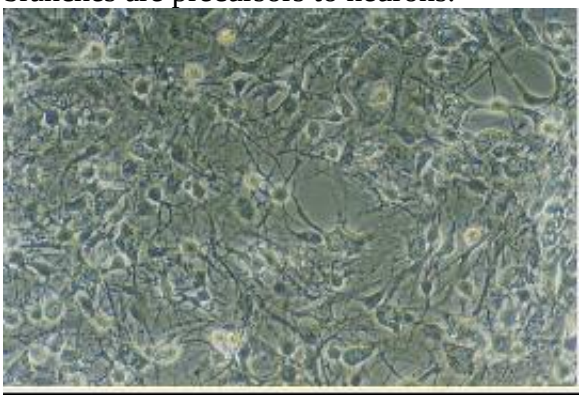
DIV 10 100X

The thick dark semi-circle of cells on the periphery of the core of the slice consists of macrophages. Macrophages and microglia can be seen establishing themselves outside of the tissue explant. There is an artifact on the right hand side of the image.



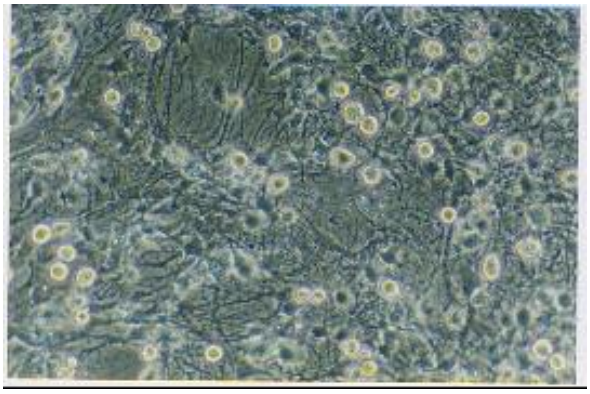
DIV 7 200X

After seven days in vitro there is already much interplay amongst the now mixed culture. This picture was taken of the cells away from the core of the slice. The brighter yellowish cells with a granular interior (nucleus) are microglia. The brownish-clear, elongated cells are fibroblasts. The dark triangular with branches are precursors to neurons.



DIV 10 400X

Here at DIV 10 neurons are in abundance. Microglia have started to differentiate into specialized cell types necessary to construct a developing neuronal network.

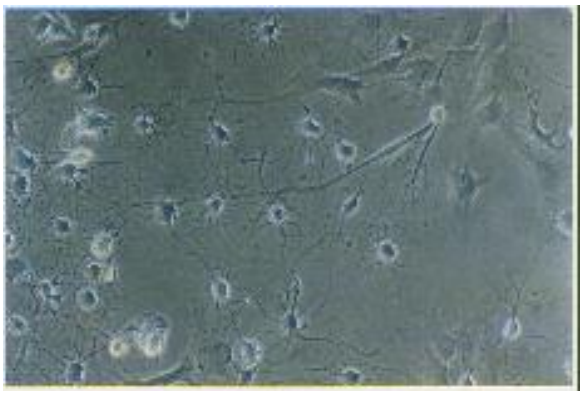


DIV 14 400X

Many undifferentiated microglia are found in this image.

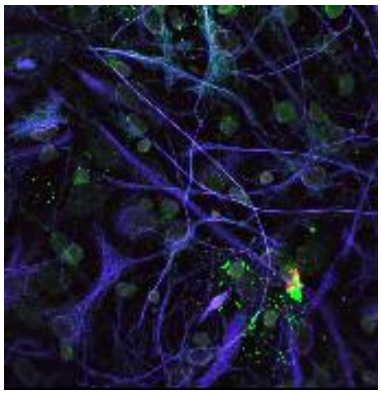
DIV 17 40X

This picture is focused to include the interface between two slices explanted to a culture dish. After 17 days in vitro large cell populations have migrated out of the explant (distinguishable by the dark brown) toward one another to create a "pancake-like" pattern throughout the well it's grown in.



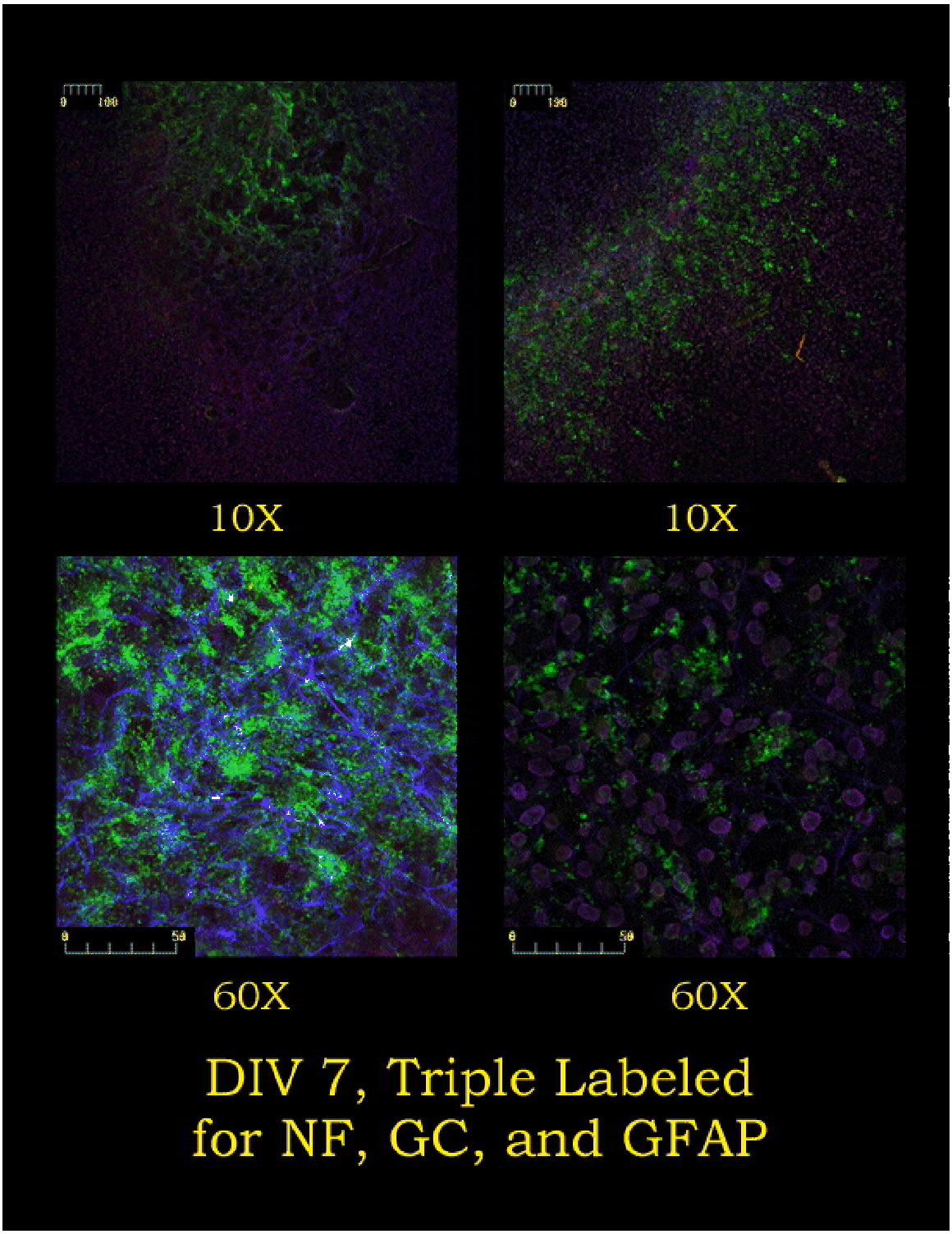
DIV 17 400X

By day 17 populations of astrocytes and oligodendrocytes have been established and are busy carrying out predicted developmental tasks. Macrophages and fibroblasts are still present as are neurons although none are present in this image.

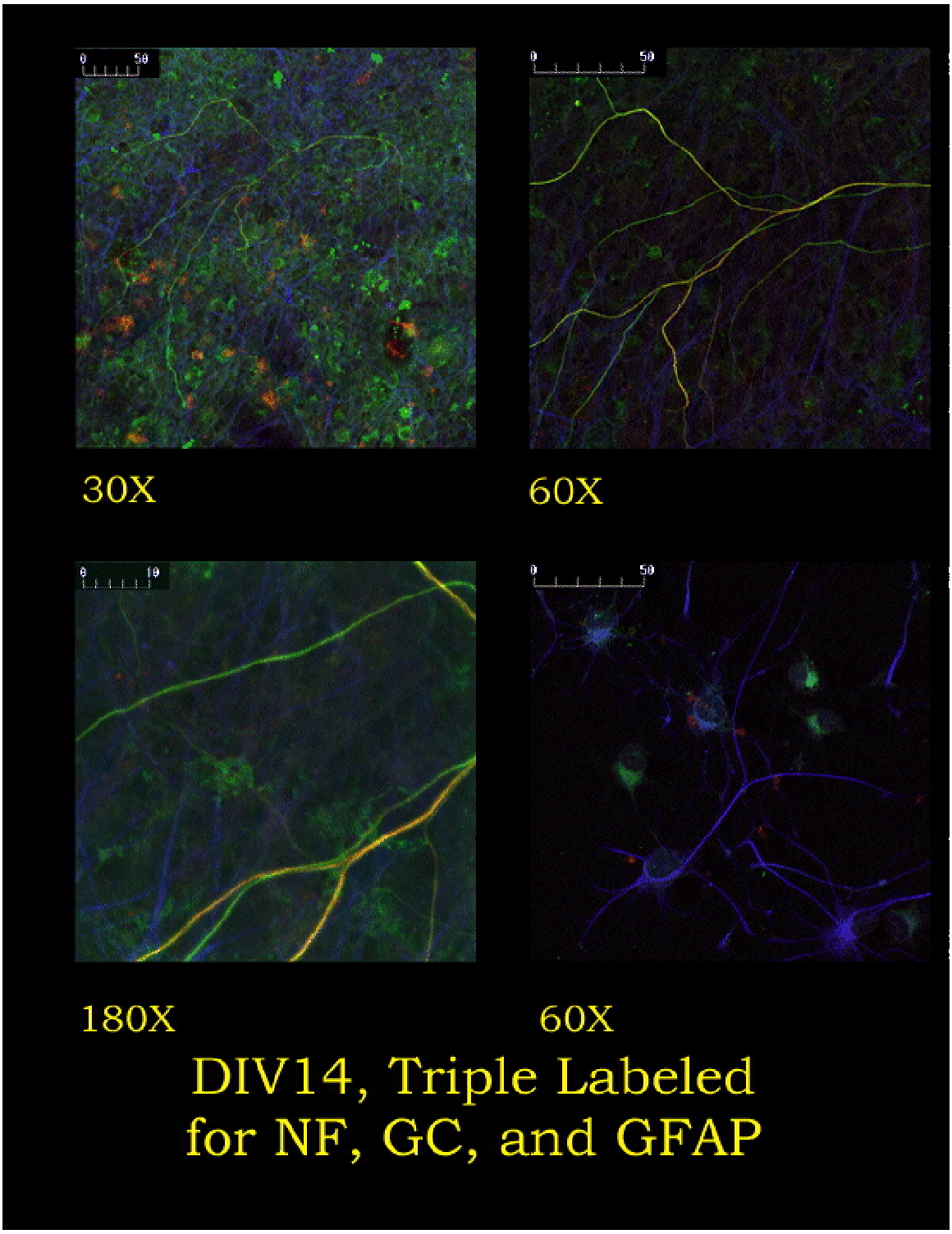


Confocal microscopy utilizes fluorescent dyes to visualize the cells in 3D whereas light microscopy is 2D. Changes in cellular columns and shapes can be monitored. We next show fluorescent confocal images of three separate days after *in vitro* culture of cerebellar formation of oligodendrocytes and myelin.

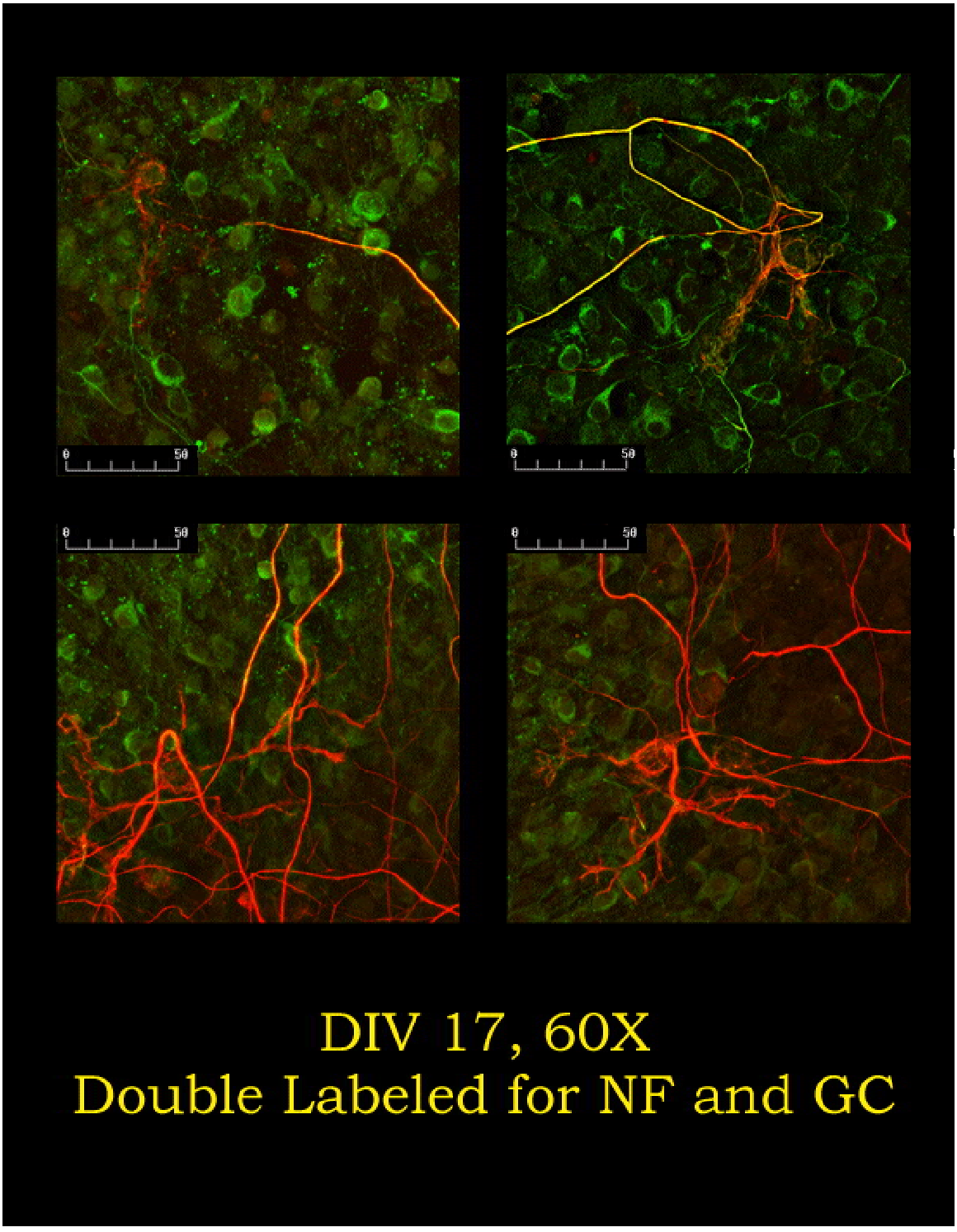
Confocal Microscopy Images of Rat Cerebellum Myelination



Confocal Microscopy Images of Rat Cerebellum Myelination



Confocal Microscopy Images of Rat Cerebellum Myelination



Discussion

In this study we used confocal and light microscopy to monitor organotypic cerebellar slice cultures from neonatal rat pups. Our images showed the migration of neural precursor cells, differentiation of these cells into glial cells and neurons, and the subsequent myelination of neuronal axons. Both light and confocal microscopy provided valuable information on morphological changes associated with neural differentiation and the formation of CNS tissue.

Light images showed the organotypic cultures growing into a sheet of cells extending from the original explant. The developing cells migrated from the periphery of the explant almost immediately after plating. By DIV 7 the cells had organized into a structural network to support the developing neurons. The precursor cells migrated and organized and subsequently differentiated into astrocytes, oligodendrocytes and neurons.

Confocal microscopy is an excellent tool for obtaining a detailed three-dimensional view of cellular structure, cellular volume and cell-cell interactions. The confocal images we generated showed the complex organization of glial cells and neurons in the slice cultures. By DIV 7 fluorescence labeling of glia such as early oligodendrocyte progenitors (Baumann and Pham-Dinh, 2001) and astrocytes are clearly visible, though developing neurons are absent. By DIV 14 developing neurons are clearly visible, astrocytes have their typical stellated structure and oligodendrocytes are maturing into what appear to be late oligodendrocytes progenitors (Baumann and Pham-Dinh, 2001). By DIV 17 mature oligodendrocytes are clearly sending out extensions of myelin, apparent as the “paisley-shaped” green structures. Clearly the oligodendrocytes proliferated rapidly with radially-extending arms that stopped when contact

was made with a neuronal axon. After contact was made, myelination proceeded, evident in the confocal images by a color change to yellow, caused by a blending of the dyes for the oligodendrocytes and neuronal axons.

There are some limitations to the organotypic method of cerebellar slice cultures. One is that the immature brain tissue is extremely fragile. Upon initial dissection care must be taken to avoid tissue damage, else macrophages proliferate and destroy the sample. The small scale of the procedure (400 μ slices) prohibits high enough yields for in-depth physico-chemical studies of the structure of myelin formed *in vitro* (Husted et al. 1994; Ohler et al, 2001; Ohler et al, 2004; Hu et al., 2004). Finally, labeling of the cells with fluorescent dyes stops the growth of the culture and thus ends the experiment at that point in time. Nevertheless light and confocal microscopy studies of organotypic cerebellar slice cultures provided confirmation of the cellular sequence of events of the formation of CNS brain tissue, including the formation of myelin.

Previous reports of cerebellar slice cultures have focused primarily on the study of neurodevelopment and physiological function (e.g., Birgbauer, Rao, Webb, 2004; Ghoumari et al., 2003). Two extensive reviews of organotypic slice cultures were written 20 years apart and their contents will not be extensively reviewed here (Seil FJ 1977; Gahwiler et al., 1997). Recent studies of cerebellar slice development utilizing immunofluorescence have focused on tissue isolated from animals at various days after birth (Casaccia-Bonnefil et al., 1999; Mathis, Collin Borrelli, 2003) as opposed to culturing the slices *in situ* as in our current study. To the best of our knowledge this is the first report of confocal microscopy of rat cerebellar slice cultures *in situ*, though numerous studies exist on confocal microscopy of oligodendrocytes and its

precursor cells (e.g., Pfeiffer SE and Warrington AE, 1994). The combined use of confocal microscopy with cerebellar slice cultures holds promise for evaluating the effects of agents that promote myelination, such as progesterone (Ghoumari et al., 2003) or other growth factors on cell-cell interaction, cell shape, cell volume and the formation of myelin. This in turn will help with understanding of demyelination and myelin repair in disorders such as multiple sclerosis and spinal cord injury.

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