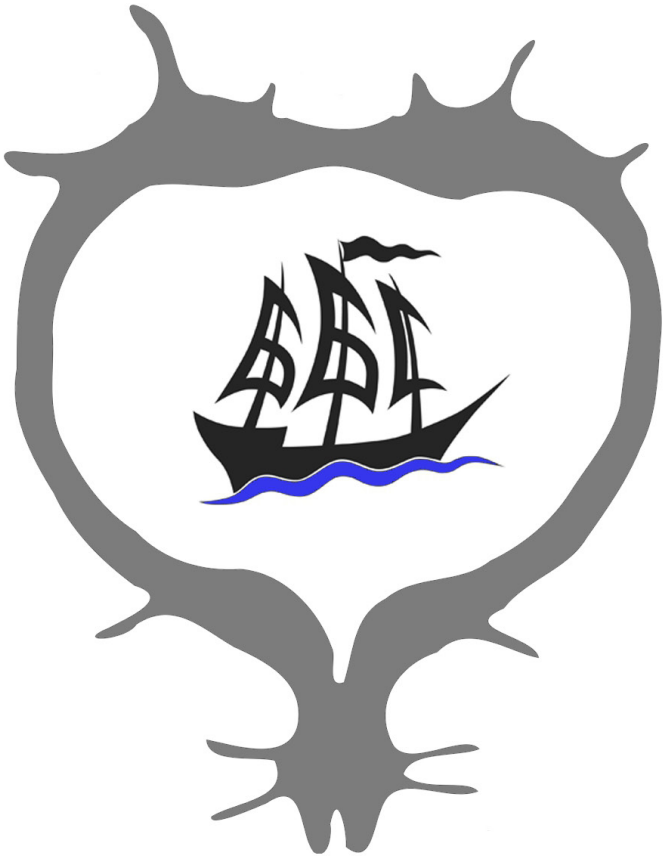


The Summer School on Comparative and Functional Neuroanatomy and Neurobiology of Invertebrates

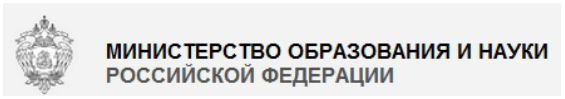
28.08 – 12.09, 2012, WSBS MSU

Lomonosov Moscow State University

White Sea Biological Station (Department of Biology Lomonosov Moscow State University)

Russian Foundation for Basic Research

The Ministry of education and science of Russia



The non-profit Dynasty Foundation

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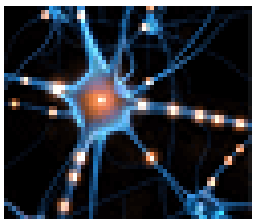

Organizing committee

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http://wsbs-msu.ru

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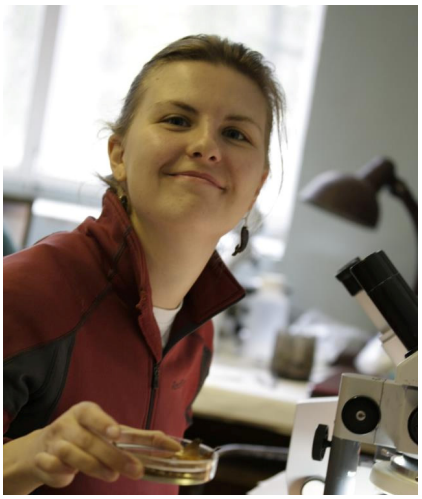

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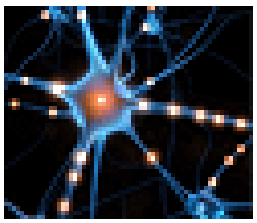


Invited professors

Dr. Sc. Leonid P. Nezlin,

Moscow, Inst. of Developmental Biology, RAS
leading research scientist;
- *expert in confocal microscopy*

nezlinl@mail.ru

Prof. Hans-Joachim Pflüger, Berlin, Germany,

Free University, Institute for Biology, Neurobiology; Methods established in Pflüger laboratory:
- *extra- and intracellular electrophysiology including neuroanatomical tracing methods*
- *(cobalt and fluorescent dyes),*
- *patch-clamp, calcium-imaging, isolated neurones, cell culture,*
- *methods of behavioural physiology (force and movement measurement)*
- *neuroanatomical methods (paraffine and plastic serial sections)*
- *immunocytochemistry, confocal microscopy including*
- *techniques of 3D analysis*

http://www.neurobiologie.fu-berlin.de/pflueger/pflueger.html

Prof. Andreas Wanninger,

University of Vienna, Austria;
Head of the Department of Integrative Zoology

http://zoology.univie.ac.at/home/

Dr. Swiddert R. Ott,

University of Cambridge, UK

- *expert in neurobiology,*
- *neuroanatomy and 3D reconstruction methods*

http://www.zoo.cam.ac.uk/zoostaff/ott.html

Dr. Thomas Stach,

Freie Universitaet Berlin, Germany

- *expert in neurobiology of Deuterostomes,*
- *4D reconstruction*

tstach@zoosyst-berlin.de

Daily schedule

Aug. 28, 2012

ARRIVAL

13:00-14:00 Lunch

14:00-16:00 Organizing accommodation and lab space

16:00-16:30 Tea

18:00-20:00 Opening of the school, Welcoming speech

20:00-21:00 Dinner

20:00-23:00 Welcome Party

Aug. 29, 2012

8:00-9:00 Breakfast (kitchen of station)

9:00-13:00 Lectures (Congress-hall, Aquarium bld.)

13:00-14:00 Lunch

14:00-16:00 Practical classes

16:00-16:30 Tea

16:30-20:00 Afternoon lecture

20:00-21:00 Dinner

21:00-22:00 Discussion/free time

Aug. 30, 2012

8:00-9:00 Breakfast

9:00-13:00 Lectures

13:00-14:00 Lunch

14:00-16:00 Practical classes

16:00-16:30 Tea

16:30-20:00 Continuation of practical classes

20:00-21:00 Dinner

21:00-22:00 Discussion/free time

Aug. 31, 2012

8:00-9:00 Breakfast

9:00-13:00 Lectures

13:00-14:00 Lunch

14:00-16:00 Practical classes

16:00-16:30 Tea

16:30-20:00 Continuation of practical classes

20:00-21:00 Dinner

21:00-22:00 Evening lecture

Sep. 1, 2012

8:00-9:00 Breakfast

9:00-13:00 Lectures

13:00-14:00 Lunch

14:00-16:00 Practical classes

16:00-16:30 Tea

16:30-20:00 Continuation of practical classes

20:00-21:00 Dinner

21:00-22:00 Discussion/free time

Sep. 2, 2012

8:00-9:00 Breakfast

9:00-13:00 Lectures

13:00-14:00 Lunch

14:00-16:00 Practical classes

16:00-16:30 Tea

Daily schedule

16:30-20:00 Continuation of practical classes

20:00-21:00 Dinner

21:00-22:00 Evening lecture/free time

Sep. 3, 2012

8:00-9:00 Breakfast

9:00-13:00 Lectures

13:00-14:00 Lunch

14:00-16:00 Practical classes

16:00-16:30 Tea

16:30-20:00 Continuation of practical classes

20:00-21:00 Dinner

21:00-22:00 Evening lecture/free time

Sep. 4, 2012

8:00-9:00 Breakfast

9:00-18:00 Excursion to the Kasian Island (with lunch)

20:00-23:00 Barbecue/free time

Sept. 5, 2012

8:00-9:00 Breakfast

9:00-13:00 Lectures

13:00-14:00 Lunch

14:00-16:00 Practical classes

16:00-16:30 Tea

16:30-20:00 Continuation of practical classes

20:00-21:00 Dinner

21:00-22:00 Evening lecture

Daily schedule

Sept. 6, 2012

8:00-9:00 Breakfast

9:00-13:00 Lectures

13:00-14:00 Lunch

14:00-16:00 Practical classes

16:00-16:30 Tea

16:30-20:00 Fishing, parasitological dissection

20:00-21:00 Dinner and fish barbecue

21:00-22:00 Continuation of discussion

Sept. 7, 2012

8:00-9:00 Breakfast

9:00-13:00 Lectures

13:00-14:00 Lunch

14:00-16:00 Practical classes

16:00-16:30 Tea

16:30-20:00 Continuation of practical classes

20:00-23:00 Goodbye Party

Sept. 8, 2012

8:00-9:00 Breakfast

9:00-13:00 Concluding remarks and student/faculty feedback

13:00-14:00 Lunch

16:00 Departure from the WSBS

Sept. 9 – Sept. 10

Train travel

Outcome of the conference

Daily schedule

Sept. 10

07:30 Arriving to Moscow

Accommodation

18:30 Public lecture of Prof. A. Wanninger at the State Darwin Museum

Sept. 11

11:00 – 13:00 Excursion to the Red Square in Moscow Downtown

Free time

Sept. 12

11:00 – 12:00 Introduction of the Moscow State University and the Faculty of Biology

12:00 – 13:00 Lunch

13:30 – 14:30 Familiarity with the Department of Invertebrate Zoology

15:00 Round table: outcome of the conference and final remarks

DAILY SCHEDULE

29/08

BASICS OF FLUORESCENCE AND LASER SCANNING CONFOCAL MICROSCOPY
Leonid P. Nezlin

PROTOCOLS OF DR. OTT AND DR. PFLÜGER (EXPLANATION)
Swidbert R. Ott, Hans-Joachim Pflüger

30/08

PROTOCOL OF DR. WANNINGER (PLANKTON)
Andreas Wanninger

BIOLUMINESCENCE OF MARINE INVERTEBRATES, MORPHOLOGY AND PHYSIOLOGY
Maria Plyuscheva

PROTOCOLS OF PHYSIOLOGISTS
Denis Abramochkin, Evgenia Volkova, Anton Zaharov

31/08

IMMUNOFLUORESCENCE STAINING IN WHOLE-MOUNTS AS A TOOL TO REVEAL THE 3D ARCHITECTURE OF THE CNS OF MARINE INVERTEBRATES.
Swidbert R. Ott

NEUROANATOMICAL TECHNIQUES FOR THE EXAMINATION OF SIMPLE NERVOUS SYSTEMS
Hans-Joachim Pflüger

PROTOCOLS OF PHYSIOLOGISTS
Denis Abramochkin, Evgenia Volkova, Anton Zaharov

Daily schedule

01/09

EVOLUTION OF THE BILATERIAN CENTRAL NERVOUS SYSTEM: A DEVELOPMENTAL PERSPECTIVE
Andreas Wanninger

EVOLUTION OF NERVOUS SYSTEMS IN DEUTEROSTOMES
Thomas Stach

PROTOCOLS OF PHYSIOLOGISTS
Denis Abramochkin, Evgenia Volkova, Anton Zaharov

02/09

INTRODUCTION TO INVERTEBRATE NERVOUS SYSTEMS
Hans-Joachim Pflüger

NEUROGENESIS OF *PHORONOPSIS HARMERI* (PHORONIDA): THE MIXTURE OF PROTOSTOMIAN- AND DEUTEROSTOMIAN-LIKE FEATURES.
Elena N. Temereva

DISCUSSION – AFTER DINNER

COMPARATIVE NEUROANATOMY IN THE AGE OF INTERNET
Thomas Stach

03/09

NOVEL TECHNIQUES IN BIOMEDICAL MICROSCOPY
Leonid Nezlin

CONTINUATIONS OF PROTOCOLS OF DR. SWIDBERT OTT AND PROF. ANDREAS WANNINGER

STUDENTS TALKS

PROTOCOL OF DR. NATALIA M. BISEROVA
HOW TO PREPARE WORMS FOR CONFOCAL MICROSCOPY? (IMMUNOCYTOCHEMICAL TECHNIQUES FOR CESTODA)

BIRD MIGRATION
Hans-Joachim Pflüger

04/09

EXCURSION TO KASIAN ISLAND. BARBEQUE
everybody

Daily schedule

05/09

NEUROGENESIS AND THE EVOLUTION OF SEGMENTATION
Andreas Wanninger

THE GOLDEN AGE OF COMPARATIVE MORPHOLOGY: LASER SCANNING MICROSCOPY AND NEUROGENESIS OF TROCHOPHORE ANIMALS
Leonid P. Nezlin

STUDENTS TALKS

TRADITIONAL FISHERY ALONG THE WHITE SEA COAST
Alexander Tsetlin

06/09

NERVOUS SYSTEM OF CESTODES: WHERE IS THE BRAIN?
Natalia M. Biserova

ALL-IN-ONE DIGITAL MICROSCOPY: A NOVEL TREND IN BIOMEDICAL RESEARCH
Leonid P. Nezlin

07/09

THE CONCEPT OF NEUROMODULATION AND ITS FUNCTIONAL ROLE FOR INVERTEBRATE BEHAVIOR
Hans-Joachim Pflüger

STUDENTS TALKS

Abstracts

BASICS OF FLUORESCENCE AND LASER SCANNING CONFOCAL MICROSCOPY

Leonid P. Nezlin

Basic principles of modern epifluorescence and laser scanning confocal microscopy will be described. Advantages and limitations of fluorescence and confocal microscopy techniques for biomedical research will be discussed and microscopes of different brands will be compared.

ALL-IN-ONE DIGITAL MICROSCOPY: A NOVEL TREND IN BIOMEDICAL RESEARCH

Leonid P. Nezlin

Recently, a new type of instruments called “All-In-One Digital Microscopes” appeared and significantly expanded the possibilities of researchers. All-in-one digital microscope systems integrate advanced imaging, measurement and recording functions into a portable, easy-to-use device. Digital imaging allows to apply online processing for image improvement: electronic phase contrast, haze reduction, quick full focus (Z-stack), image stitching, advanced navigation system, long term live-cell imaging, and many more. The systems manufactured by Keyence Corporation (Japan) will be described.

THE GOLDEN AGE OF COMPARATIVE MORPHOLOGY: LASER SCANNING MICROSCOPY AND NEUROGENESIS OF TROCHOPHORE ANIMALS

Leonid P. Nezlin

Immunochemical techniques and laser scanning confocal microscopy have considerably expanded the capacity of comparative morphology and allowed us to study neuronal development of trochophore animals at the level of individual identified neurons. It proved that some generally accepted concepts of the larval nervous system and phylogenetic theories constructed on this basis are incorrect. Comparative analysis has shown that the orthogonal brain is absent at all developmental stages in the representative Lophotrochozoa members. Fundamental differences in the structure and development of the nervous system in trochophores that belong to different taxonomic groups within Lophotrochozoa have demonstrated that the trochophore larvae in these groups are not homologous, and their similarities most likely result from convergence. Our results challenge the concept of trochophore as the ancestral form common for all trochophore animals. It is necessary to exclude from phylogenetic discussions the orthogon as a basic plan for the structure of the nervous system and the trochophore as an ancestral form for all Lophtrochozoa.

NEUROANATOMICAL TECHNIQUES FOR THE EXAMINATION OF SIMPLE NERVOUS SYSTEMS

Hans-Joachim Pflüger

In this lecture several methods of functional neuroanatomy will be discussed: (i) Methods that are useful for an overall-examination of peripheral nervous systems such as Methylenblue or central nervous systems such as silver- (Golgi, Bodian) or osmium-staining (Ethygallate); (ii) methods that are used in functional neuroanatomy such as antero- and retrograde axonal tracing (cobalt, fluorochromes), intracellular dye injection, or immunocytochemistry; (iii) methods that are used in data anylysis (camera lucida, confocal microscopy).

THE CONCEPT OF NEUROMODULATION AND ITS FUNCTIONAL ROLE FOR INVERTEBRATE BEHAVIOR

Hans-Joachim Pflüger

Abstract of lecture on Neuromodulation

This lecture will first give a definition of neuromodulation, and subsequently give examples on the importance of neuromodulation, for example (i) for the configuration of functional networks from anatomical networks, (ii) for the development of networks, (iii) for the regulation of synaptic transmission and energy metabolism, and (iv) for the regulation of whole behavioural states such as aggression. Several invertebrate systems ranging from molluscs, annelids and crustaceans to insects will be examined.

INTRODUCTION TO INVERTEBRATE NERVOUS SYSTEMS

Hans-Joachim Pflüger

Abstract of lecture on Biogenic amines

Biogenic amines are most important for both the generation and control of behaviour. Invertebrate systems have enormously contributed to the knowledge on neuromodulatory function. In this lecture the different roles of tyramine and octopamine in the insect nervous system will be considered and compared to their roles in the nervous system of other phyla.

NEUROGENESIS OF *PHORONOPSIS HARMERI* (PHORONIDA): THE MIXTURE OF PROTOSTOMIAN- AND DEUTEROSTOMIAN-LIKE FEATURES.

Elena N. Temereva

The investigation of nervous system development and organization is the most important criterion allowed to establish the relationship between large taxa. According to recent data, the neurogenesis and nervous elements organization are different in protostomian and deuterostomian. Phoronida is the small phylum, which is traditionally regarded as deuterostomian. However, according all molecular data, phoronids are prtostomian

animals, which are closed related to typical Spiralia. The study of phoronid neurogenesis may shed a light on the problem of phoronid phylogenetic relationship. Using immunecytochemistry and confocal microscopy, we investigated the development of the serotonin-like and FMFRamide-like immunoreactive nervous system during larval development of *Phoronopsis harmeri* Pixel, 1912.

The first neurons are serotonergic; they appear in the epidermis of the apical plate. In the young larva of *Phoronopsis harmeri*, the serotonergic nervous system consists of apical ganglion, which contains a U-shaped field of monopolar perikarya, and two groups of bipolar (or multipolar) perikarya; the medial and marginal nerves of the preoral lobe; tentacular neurite bundle, which runs dorsally from the left and right groups of bipolar (or multipolar) perikarya; two nerve rings of the telotroch; the oral nerve ring; the nervous net around the proctodaeum and pyloric sphincter; and the ventral nerve cord, which consists of two longitudinal neurites with repetitive paired neurons contacted each other via repetitive commissures. In advanced larvae, some of these elements disappear. The FMRFamide-reactive nervous system appears at later stages than the serotonergic nervous system. At early stages, FMRFamide-reactive neuritis is evident in the epidermis of the apical plate. Then first FMRFamide-reactive perikarya originate on the dorso-lateral sides of the preoral lobe near the apical plate. With age, these perikarya increase in number. In opposite to other phoronid larvae, *P. harmeri* larvae have numerous FMRFamide-reactive perikarya in the apical organ, in the epidermis of the preoral lobe, in the epidermis of the ventral and ventro-lateral sides of the oral field (collar region), and in the epithelium of the esophagus and the midgut. In the apical organ of older larvae, FMRFamide-reactive perikarya form two dorso-lateral groups of monopolar and multi- or bipolar neurons. Six- and nine-day-old larvae have a medioventral nerve cord, which extends from the oral nerve ring to the tentacular neurite bundle and composed of longitudinal neurites with repetitive paired neurons contacted each other via repetitive commissures. The FMRFamidergic nervous system is very complex; the main nervous tracts underline the main muscles.

P. harmeri has the most complex larval serotin- and FMRFamide-like immunoreactive nervous systems of all phoronids studied to date. The gross anatomy of the nervous system of the actinotroch larva combines characteristics of both lophotrochozoan and deuterostome larvae. On the one hand, the phoronid apical organ consists of flask-shaped serotonin-like immunoreactive monopolar perykaria, which most likely constitute a lophotrochozoan apomorphy [8]. On the other hand, the complexity of the phoronid apical organ, which contains more than 40 serotonin-like immunoreactive perikaria, resembles that of some deuterostome larvae. The distant relationship of phoronids and deuterostomes suggests independent origin of these complex apical organs, although a complex apical organ at the base of the protostome-deuterostome split with subsequent independent simplification in most spiralians cannot be ruled out. The finding of a paired ventral neurite bundle with serially arranged commissures suggests that such a neural feature was part of the ancestral phoronid – and most likely also the ancestral lophotro-

chozoan – bodyplan, which was secondarily lost in adult phoronids, probably in connection with the acquired sedentary lifestyle.

IMMUNOFLUORESCENCE STAINING IN WHOLE-MOUNTS AS A TOOL TO REVEAL THE 3D ARCHITECTURE OF THE CNS OF MARINE INVERTEBRATES.

Swidbert R. Ott

Confocal microscopy enables the analysis of specimens several hundreds of micrometres thick and has therefore become an important technique in the functional and comparative neuroanatomy of invertebrates. The fixed but otherwise intact central nervous system can be stained by immunofluorescence using antibodies against a wide range of molecules such as neurotransmitters or signalling proteins, and the distribution of the target molecule can then be analysed in confocal optical sections obtained from this whole-mount preparation. Compared with cutting physical sections, confocal microscopy greatly reduces the labour required to obtain complete series, and three-dimensional volume reconstructions are readily generated, since confocal sections are perfectly aligned and already in digital format.

In this workshop, we will combine methodological theory and hands-on neurobiological research. I will introduce you to the theoretical background and practical considerations and limitations of the whole-mount immunofluorescence technique. This will include ways to overcome three major technical challenges: limited penetration of antibodies, loss of antigenicity, and tissue opacity.

We will then use the technique to compare the neuroarchitecture across different groups of marine invertebrates and relate it to their sensory ecology and behavioural requirements. An effective marker for revealing the overall organisation of the CNS is immunostaining against synapsin. Synapsins are phosphoproteins that associate selectively with small synaptic vesicles and are therefore expressed abundantly in regions of high synaptic density (synaptic neuropiles). We will combine anti-synapsin staining with staining against other neural signalling molecules to generate multi-labelled preparations.

EVOLUTION OF NEURAL CIRCUITS IN OPISTHOBRANCHIA (MOLLUSCA)

Paul S. Katz

Neuroscience Institute, Georgia State University, Atlanta, GA, USA

This lecture will discuss the history of research using identified neurons in Opisthobranchs. These animals include: *Aplysia californica*, *Tritonia diomedea*, *Melibe leonina*, and *Clione limacine*. We will go over some basic neural circuitry underlying behaviors such as swimming. We will discuss the neural mechanisms underlying these behaviors

and computational models of the neural circuits. Finally, we will compare neural circuits across species and discuss the evolution of these circuits.

EVOLUTION OF THE BILATERIAN CENTRAL NERVOUS SYSTEM: A DEVELOPMENTAL PERSPECTIVE

Andreas Wanninger

The recent years have seen a dramatic increase of data concerning the anatomy and development of the nervous system in hitherto little investigated metazoan groups, which is mainly due to the establishment of fluorescence antibody staining in combination with confocal microscopy and 3D reconstruction techniques as routine research tools. These novel data may now be used for phylogenetic analyses - an approach sometimes referred to as “neurophylogeny” - but also provide new input for the discussion on various aspects of the evolution of animal nervous systems. In this lecture, some of these recent data will be presented in an evolutionary and phylogenetic context and possible scenarios for the evolution of the central nervous system in Bilateria will be discussed.

NEUROGENESIS AND THE EVOLUTION OF SEGMENTATION

Andreas Wanninger

Most animals are characterized by a serial arrangement of various organ systems (e.g., nephridia, muscular or neural components) along (parts of) their longitudinal body axis. While overall similarity in the arrangement of, in particular, neuromuscular structures such as bodywall ring muscles or repeated commissures and ganglia exists between clades, the way as to how these structures are formed are strikingly different. Some groups, such as annelids or arthropods, show a directional mode of formation from a posterior proliferation zone, others lack such a predictable ontogenetic process of subsequently formed structural units. Contrary to the latter, the former is usually considered as being associated with a so-called “segmented” bodyplan, although detailed comparison shows that differences exist in the way as to how these growth zones function in the individual segmented clades. In this lecture, examples how data on invertebrate neurogenesis may help to infer remnants of ancestral segmentation in seemingly non-segmented animals will be presented and alternative hypotheses concerning the evolution of segmentation will be discussed.

EVOLUTION OF NERVOUS SYSTEMS IN DEUTEROSTOMES

Thomas Stach

While Deuterostomia comprises merely 60 000 species, these species display an astonishing diversity in anatomy and life histories. Phylogenetic relationships among higher deuterostome taxa are not resolved. Molecular systematic phylogenies are contradictory and plagued by high mutation rates in tunicates and especially neurophylogenetic hypotheses contradict molecular systematic hypotheses. In my lecture I emphasize

the necessity of a comparative paradigm to phylogenetic argumentation. I demonstrate advances in our understanding of morphogenesis of nervous systems in deuterostomes and their contribution to phylogenetic inferences. Examples will include comparative immunohistological studies, comparative anatomies using 3D-reconstructions, and analyses of processes of neurulation in chordates and enteropneusts. In chordates our knowledge of neurulation is considerably more detailed than in enteropneusts and can be analyzed on the single cell level within a fate map approach. It is even possible to utilize such detailed information to infer sister group relationships. Homology hypotheses are shown to be at the center of all phylogenetic and evolutionary research and I discuss methods how character conceptualization can be achieved. The internet allows researchers to internationally cooperate in this endeavor and simultaneously allows for a considerably improved standard of data documentation in comparative morphological research. I will demonstrate MorphDBase as a valuable online tool to this end.

BIOLUMINESCENCE OF MARINE INVERTEBRATES, MORPHOLOGY AND PHYSIOLOGY

Maria Plyuscheva

Bioluminescence occurs in many different species in phylogenetically diverse groups. The type of light and the emission methods, as well as the color, may be very different. The functions may differ among organisms but also a given organism may utilize luminescence in more than one way. Bioluminescence functions may be classified under three major categories: defensive (as a help to escape from predators), offensive (as a support for predation), and communication (for courtship or mating). Within each category a number of different specific strategies are recognized. For instance, luminescence may be used as a diverting decoy, as a frightening flash, or as a shape camouflage using ventral luminescent spots.

The biochemistry of luminous systems is known in detail only for bacteria, dinoflagellates, cnidarians, and fireflies, although some information exists for another half-dozen or so luminescent, the polychaetes among them. However, this phenomenon occurs sporadically in unrelated polychaete species having different modes of life and belonging to different families, such as the Aphroditidae, Tomopteridae, Syllidae, Alciopidae, Chaetopteridae, Cirratulidae, Terebellidae, and Polynoidae.

In some species of the scale-worm sub-family Polynoinae (*Harmothoe* Kinberg, 1856) and sub-family Acholoinae (*Acholoe* Claparede, 1870*)* an area of the epithelium of the lower surface of the elytra has been reported to emit light flashes upon stimulation. Some other polynoids seem not to be luminescent (e.g., *Lepidonotus clava* Montagu, 1808*, L. squamatus* Linnaeus, 1767*, Halosydna gelatinosa* Sars, 1835*, Lepidasthenia argus* Hodgson, 1900). In the light-producing species, the lower surface of the elytra has a layer of luminescent cells or photocytes (actually modified epidermal cells), which are lacking in the non-luminescent species (Nicol 1953). The behavioral or ecological func-

tion of this bioluminescence remains unknown, although it has been suggested that the signal could be either a warning or a distracting mechanism.

NERVOUS SYSTEM OF CESTODES: WHERE IS THE BRAIN?

Natalia M. Biserova

The lecture goal is: 1) to summarize our knowledge about cestodes nervous system; 2) to understand what correct terminology we can use to describe of nervous system in cestodes.

What we know about cestodes brain?

The “pseudophyllidean” model of the cestodes brain consists of two lateral lobes with two neuropiles connected by the single median commissure; part of cestodes have unpaired central lobe into the brain.

Trypanorhincha model of the cestodes brain includes nine neuropiles and three types of commissures: median, crisscross and anterior semiring commissures, 4 paired lobes and one unpaired lobe.

At least two groups of cestodes lack the median commissure and lobes in “the brain”: Caryophyllidea and Spatobothriidea.

Such group as Amphilinidea has more concentrated nervous system with metamerism features. Amphilinids have an asymmetrical position of two cerebral ganglia and many ganglionar knots in main cords and also has several well-developed caudal ganglia.

Cestodes nervous system and especially brain structure is varying in different orders and often disparate with other invertebrates.

To avoid the accumulated contradictions, it is necessary to build a logical chain of the complicating of the nervous system, not evolutionary, but logical. For example: Neuron → Neuropil (compact concentration of neurites with synapses) → Ganglion: somata with neuropil (no neuropil – no ganglion) → Commissures and connectives → Brain (organ of adult animal; it includes neurons, neuropils, commissures and further types of cells, like glial cells).

Young scientist division

NEW DATA ON THE NERVOUS SYSTEM OF THREE PHYLACTOLEMATE SPECIES: *CRISTATELLA MUCEDO*, *PLUMATELLA REPENS* AND *FREDERICELLA SULTANA*

Ksenia Shunkina

New data concerning the details of organisation of the phylactolaematous bryozoa nervous system are presented. We have used CLSM methods to visualize the general structure of the nervous system and also 5HT- and FMRFamid-positive parts of the nervous system. It was shown that the general structure of the central nervous system ap-

pears to be the same in all three investigated species. Some differences were found in the organisation of lophophore innervation. 5HT-positive part of the nervous system is presented by a neuropil in the cerebral ganglion region. FMRFamid-positive part is presented by an assemblage of neuron bodies on the lower part of the cerebral ganglion. All the differences in the nervous system structure are considered to be dealt with the size of the lophophore.

THE PECULIARITIES OF BRAIN STRUCTURE AND ULTRASTRUCTURE OF THE SMALLEST INSECTS AS A RESULT OF MINIATURIZATION

Anastasia Makarova

Minituarisation is one of the main trends in the morphological evolution of the insects. The extreme small size of the body influences the morphology, physiology and life history. Under the decrease of the body size the essential transformation of all systems of organs occur. Conserning nervous system, oligomerization and concentraition of ganglion and decrease of neurons size take place. The three-dimensional computer reconstractions were obtained and relative values of brain and some of it characters were analysed. The new obtained data about the number and size of neurons in the brain of Ptiliidae and Mymaridae confirm and complete the hypothesis about the factors limiting miniaturization of insects. The prominent transformation were found: the decrease of the diameter and increase of the neurons density. Due to the extreme conservatism the important limitation of the decrease of the body size consists in the size of the nervous system, on the one hand, and size of neurons and diameter of the axons from the other hand.

BRAIN AND NERVE CORD OF VESTIMENTIFERA (ANNELIDA): COMPARISON WITH POLYCHAETES

Nadezda Rimskaya-Korsakova

Vestimentifera is a group of huge gutless tubeworms inhabiting reducing environments of the World Ocean. The worms belong to polychaete group Siboglinidae (or Pogonophora) that has not worldwide accepted position within Annelida (Liwanow, Porfirjewa, 1967; Bartolomaeus, 1995; Black et al., 1997; McHugh,1997).

Recent molecular phylogenetic research proves the close relationship between Siboglinidae and polychaetes Oweniidae (Rousset et al., 2004) and possible sister groups “Pogonophora-Oweniidae” and “Clitellatamorpha” (Zrzavý et al., 2009). One of principal morphological feature combining Siboglinidae and Oweniidae is intraepidermal nervous system (Ivanov, 1960; Liwanow, Porfirjewa, 1967; Bubko, Minichev, 1972). However, Oweniidae is not the only polychaetes with nervous system inside epidermis. While there is no data on nervous systems organizations of these group of worms, we hardly are able to compare Oweniidae and Siboglinidae. Here we present results of studing of ner-

vous system of vestimentiferans *Riftia pachyptila, Oasisia alvinae* and *Lamellibrachia satsuma*.

5 specimens of R.pachyptila (from hydrothermal vents at 21°N and 9°N on the East Pacific Rise and at Guaymas Basin in the Gulf of California), 2 specimens of O.alvinae (21°N, East Pacific Rise) and 1 specimen of L. luymesi (Kagoshima Bay, Japan) were used. Two former species were collected by DSV Mir-I and Mir-II during the 49th cruise of R/V Akademik Mstislav Keldysh in 2003. The latter one was kindly provided by K. Fujikura of JAMSTEC.

Standard histological methods were applied for studing the morphology of the central nervous system.Sections of R.pachyptila were used in three-dimensional reconstructions with commercial software 3D-DOCTOR 3.5.040724 (Able Software Corporation of Lexington, MA, USA). 19 objects were traced on 77 sections of the brain. The field of view was of 2812 μm, producing a voxel size of 0,721 x 0,721 x 15 μm^3.

Vestimentiferans have intraepidermal, unpaired, non-ganglionated ventral nerve cord. Only in the vestimentum the cord is paired. Here the components of the cord are connected by numerous commissures . Two anterior segments have numerous segmental nerves that branch off from the cord and form anastomoses. In opisthosome there is 1 segmental nerve per segment .

For the first time brain of Vestimentifera was 3d-reconstructed. It is massive, ventral, completely intraepidermal, laterally bilobed structure. For the first time 2 pair of perikarya of giant axons were detected. It consists of merged supraesophagial and subesophagial ganglia (parts of the brain that situated dorsally and ventrally to reduced gut, respectively). The ganglia are connected by paired *longitudinal nerve tracts* which run forward from ventral nerve cord and connect by two transverse commissures (*dorsal and supraenteral ones*). The *longitudinal nerve tracts* and *the dorsal and supraenteral commissures* can be generally homologized with circumesophagial connectives and their dorsal and ventral roots of polychaetes. Vestimentiferan tentacular nerves come from *lateral zones of longitudinal nerve tracts*. So, innervation of vestimentiferan tentacles allow us to suppose their homology with polychaete palps . In contrast, obturacules (peculiar morphological feature separating Vestimentifera from other Siboglinidae) are innervated from *dorsal comissure* that gives us prove that they are not homologous to vestimentiferan tentacles and polychaete palps .

In comparison with ground pattern of nervous system of Polychaeta Vestimentifera combines primitive features of Oweniidae (intraepidermal location, numerous commissures, numerous segmental nerves and nervous plexuses) and compound brain of typical polychaetes. The latter one is a result of large tentacular crown and huge obturacules. Modern investigations of different patterns of annelid nervous system prove that structure of vestimentiferan nervous system corresponds and does not go beyond the limits of annelid nervous system organization.

We thank the captain and crew of R/V *Keldysh* and crew of DSV *Mir*. Special thanks are also due to L.I.Moskalev, A.V. Gebruk, E.N.Temereva, L. Rusin, G.P.Salkova for their enthusiasm and assistance. We thank K. Fujikara for providing specimens of *L.satsuma*.

THE FINE STRUCTURE OF SOME POLYCHAETES' EPIDERMIS (FAM. OPHELIIDAE, SCALIBREGMATIDAE AND FLABELLIGERIDAE)

Stepan Vodopyanov

There is one special case of the epidermis structure within Polychaeta – the "stratified" integument of opheliid *Travisia forbesii* (Storch, 1988). The description of its structure is not quite clear yet, there are several questions to solve: where is the basal lamina, how do the cells are connected with neighbors and with the basal lamina, what are the great intercellular spaces, what is the thick collagen layer below the epidermal cells. On the other hand most of the species of the genus Travisia, including *T. forbesii*, bear the epidermal papillae on the body surface (Kudenov, 1975, Dauvin, Bellan, 1995). There is also the discussion among molecular phyllogenists about the position of the genus Travisia and the proposal to transfer it from family Opheliidae to family Scalibregmatidae (Bleidorn et al, 2003, Paul et al, 2010). We are studying the epidermal papillae of *Travisia forbesii*, the epidermis of *Scalibregma inflatum* (Scalibregmatidae) and *Ophelia limacina* (Opheliidae) and also trying to compare Travisia's epidermis with the epidermal papillae of *Brada inchabilis* and *Flabelligera affinis* (Flabelligeridae).

The epidermis of *Travisia forbesii* is built in such way: the outer most layer is the cuticle and there are **spherical papillae** embedded within and surrounded by it. They are tightly packed together. There is an **inner cuticle layer** under the papillae. Each papilla has a **peduncle**. It runs through the inner cuticle layer and connects the papilla with the **epidermal cells** below. These cells rest upon **basal lamina** – an extracellular matrix layer, which borders the epidermis and the body wall musculature. All the cells are connected by apical cellular junction complex – all around the papilla The wide intercellular electron-transparent spaces remain instead of empty glandular cells - they died after discharging their secretions. The peduncle of the papilla is constructed by only one cell. It has a shape of a hollow cylinder. It is C-shaped in the cross section and has fissures on the inner side. Bundles of some slender cellular projections run along these fissures. We need to prove they are nerves with immunocytochemistry. The neighboring cells to the peduncle are also connected to the peduncle cell – above and below the inner cuticle layer.

A peduncle has a core – it is a high fold of the basal lamina. The peduncle cell contains many bundles of tonofilaments – they are connected to the core with basal hemidesmosomes. The core has a pyramid-like extension on its upper part and several slender projections run inside the papilla out of this extension. The epidermal cells under the inner cuticle layer have flattered shape with many interdigitations. They are mixed with bundles of rich intraepidermal nervous plexus presumambly. We'd like to call the

epidermis of *Travisia forbesii* pseudo stratified – because the peduncle joins the cells of the papillae and the rest ones.

Scalibregma inflatum is quite different. *Scalibregma* lives inside U-shaped tube like a lugworm within the muddy sediment of the upper part of sublitoral. The SEM photos show us the same somehow square block arranged in the rows. The sections reveal the glandular cell mostly in a line, one by one. No inner cuticle neither, no peduncles there. The basal lamina goes somehow folded but we have no a lot of TEM sections of *Scalibregma* so far unfortunately.

Almost whole surface of *Ophelia's* body is smooth. There are some bumps on the lateral sides of the posterior half of the body. But they are not glandular papillae. The diagonal muscle cells join to the thick cuticle through the basal lamina and the tendon epidermal cells there and such connection bends the cuticle – this bumps arise – pulling the cuticle inwards.

Just a few data we have about flabelligeridae polychaetes so far. They are famous for their tunic – mucous or built with sand grains and diatoma fragments. Some *Brada* has big hemispheric organs. Borodin in 1929 described the in detail for a couple *Brada* species. One the scheme from the Borodin article looks quite similar to travisia's papillae. The narrow outgrowths of ECM and basal lamina also build the supportive structures in the thin parts of epidermal organs. But several cells surround them there. *Flabelligera affinis* bears not sand grains, but mucous tunic. The sections of the epidermis look quite different. And it is hard for us to find basal lamina there and the cells borders so far.

New morphological data should be used for new phylogenetic trees of Polychaeta.

Is there any correlation between the way of life (within the sand, mud, on the surface or inside the sediment, the way how worms move) and the structure of the skin? - Yes, but how it is possible to prove? Is it convergence or homology? I'd like to thank my supervisors, teachers; all people helped me with electron microscopes.

THE STRUCTURAL ORGANIZATION OF NERVOUS SYSTEM SOME NEMERTEANS SPECIES FROM WHITE SEA.

Sergey Petrov

Organization of nemerteans nervous system have been studied in Poseidon (Lineus) ruber, Malacobdella grossa, Tetrastemma candidum and Cephalotrix linearis from different orders, which didn't described early. We used method of silver impregnation by Golgi-Collonier, histochemical method of detection catecholamine with glyoxylic acid. We described a structural organization of the central and peripheral nervous system, described the shape, density, location and relationship of bodies and processes of neurons. We showed organization of nervous plexus in the gut, proboscis, sucker, rinhocoel, body wall and blood vessels. We detected a lot of intra- and sub-epithelial receptors. Sensory cells had different shape and different number and location of processes in sensory organs and along the surface of epithelium. Some groups of neurons have been described in CNS. Differences catecholaminergic elements in peripheral and central nervous system were described in examined nemerteans species.

Practical courses

For training and performing the practical tasks, students will be divided into 4 groups of 3 persons each. Each group will pursue an independent task, and each group will execute all tasks within 12 days. Special time is reserved for the presentation of results by students, and for evening discussions.

PART I. FUNCTIONAL NEUROANATOMY *(L. NEZLIN, N. BISEROVA, H-J. PFLÜGER, , T. STACH, S. OTT)*

1. Multi-label immunostaining techniques.

Participants will learn in detail how to select primary and secondary antibodies, the strategy of fixation and antigene retrieval, immunostaining, embedding, data aquisition and processing.

2. Tracer injection techniques in neuromorphology and neurophysiology.

Participants will learn the technique of back-filling (tracer injection into the nerve) and front-filling (tracer injection into the cell body) using various tracers and their visualization with fluorescent markers.

PART II NEUROPHYSIOLOGY *(D. ABRAMOCHKIN, A.M. ZAKHAROV, E.P. VOLKOVA)*

3. Registration of miniature end-plate potentials in longitudinal muscle fibers of the lobworm *Arenicola marina*.

4. Analysis of the regulation of calcium-dependent luminescence in the polychaete *Harmothoe imbricata.*

SUGGESTED EXPERIMENTS FOR PRACTICAL COURSE OF PROF. PFLUEGER

(1) Axonal diffusion techniques for tracing neurons

This is a method that applied anterogradely or retrogradely will either reveal the distribution of peripheral receptors associated with a particular nerve and the innervation patterns of muscles, glands and other target organs, or reveal the distribution of motor neurons including their dendritic trees and sensory axon terminals (axonal trees) within the CNS. This is a powerful method of functional neuroanatomy that allows mapping the locations of neurons or sense organs and, in connection with staining architectural features of the CNS, is the first step in the study of a functional organization of the CNS.

(2) Immunocytochemical examination of peripheral and central nervous systems

Antibodies to respective antigens with subsequent intensification by fluorochrome-labelled secondary antibodies can be used to map the distribution of transmitters, enzymes or ion channel components (subunits) both in the peripheral and central nervous system.

(3) Analysis with confocal microscopy and reconstruction programs

Image stacks of optical sections of the CNS, gained on the confocal microscope, will be used for further analysis in reconstruction programs such as Amira or ImageJ.

DEXTRAN AXONAL TRACING („BACKFILL“)

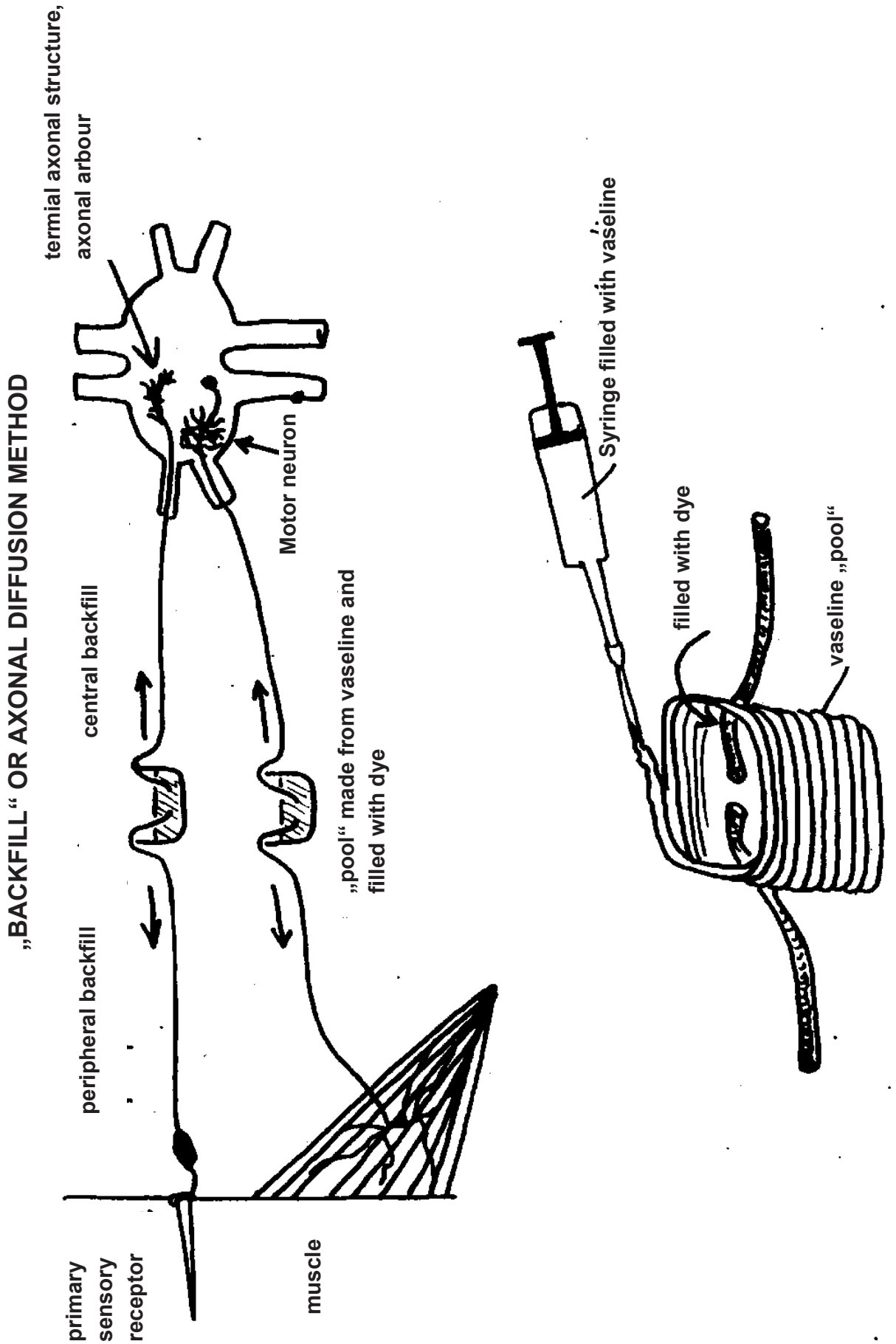
Hans-Joachim Pflüger (pflueger@neurobiologie.fu-berlin.de)

- Backfill with Dextran (tetramethylrhodamine 3000MW, Invitrogen D 3308, dissolve some crystals in 100µl A.dest until color is “Bordeaux”-red)
- Leave preparation in saline in a moist chamber in the refrigerator (over night or longer)
- Remove petroleum jelly („vaseline“) pool and remove dye with tissue
- Wash with saline
- Fix in 4% Paraformaldehyde (PFA) in 0,1M PBS at room temperature for 2 h (shaker)
- Wash 3 × 15 min in 0,1M PBS
- Dehydrate in ascending ethanol series (50%, 70%, 90%, 100%), 10 min each
- Ethanol: methyl salicylate mixture 1:1, for 10 min.
- Clear in pure methyl salicylate, 10 min.
- Embed on slide in methyl salicylate
- Confocal Microscope, 543 nm excitation

AXONAL DIFFUSION METHODS („BACKFILL-TECHNIQUE“, AXONAL TRACING)

- anterograde or retrograde axonal diffusion
- dyes diffuse through cut axons; cut nerve end is placed into a solution of dye
- Diffusion time depends on distance and, thus, size of the organism, and ranges from hours (Drosophila) to several days (for example filling large projection neurons extending over several ganglia)
- Dyes can be fluorescent dyes (for example dextranes to which fluorochromes are coupled, for example, dextrane-rhodamine), or cobalt or nickel salts which then have to be precipitated by sulfide ions (either H_2S or $(NH_4)_2S$) or by rubeanic acid (gives coloured precipitation products)

Protocols


„BACKFILL“ OR AXONAL DIFFUSION METHOD
termial axonal structure,
axonal arbour
central backfill
Motor neuron
peripheral backfill
„pool“ made from vaseline and
filled with dye
primary
sensory
receptor
muscle
Syringe filled with vaseline
filled with dye
vaseline „pool“

PROTOCOL OF DR. SWIDBERT OTT

(Crustacean: isopoda, decapoda)

Wholemount Protocol — time-table overview:

This is already with reduced incubation times in the antibodies (pAB: 2.5 days, sAB: 1.5 days). If we were to start dissections on the 29th we would win a day but it would leave us little time to familiarise us with the fauna.

- **Day 1**: 30.08. dissections, start fixation (over night)
- **Day 2**: 31.08. fine-dissection of fixed CNS, permeabilisation (Dent's), get into pAB by late afternoon: for 2.5 days, pAB would be until Day 5
- **Day 5**: 03.09. wash and start sAB: for 1.5 days, sAB would be until Day 7.
- **Day 7**: 05.09. wash, dehydrate
- **Day 8**: 06.09. mount and confocal analysis
- Then 07.09. more confocal microscopy and analyse data

Protocol: wholemount immunofluorescence staining of invertebrate CNS

Incubation times as used in Ott 2008 J. Neurosci. Methods for adult locust brains. For smaller objects, or if not feasible otherwise, incubation times can be shortened. Simpler alternatives that give comparable results are given for rehydration (step 6) and final dehydration (step 12).

Steps (**2)** and (**4–7**) are carried out in ~10 ml capacity glass vials (~2 cm diameter, 3 cm tall) with plastic snap-on lids.

Ott 2008 used HEPES-buffered saline in step (**1**) and (**3**) but HEPES is quite expensive and not necessary; replacing HEPES with Tris is fine.

1. Expose brain under physiological Tris-buffered saline (TBS).

- If phosphate-buffered saline is used, water-insoluble zinc phosphate will precipitate when passing the tissue into ZnFA.

2. Fix in situ for ~20 h at room temperature in ZnFA fixative under agitation.

- Match osmolarity of ZnFA to that of the saline by using NaCl to balance tonicity with respect to inorganic ionic solutes and sucrose for larger organic solutes; formaldehyde is *not* included in the calculation.

3. Dissect out brain under TBS.

- When dissecting, ZnFA-fixed tissue handles differently from PFA-fixed tissue: it is brittle and tolerates little stretching, but different organs and tissue elements readily separate along their boundaries.
- Carry out steps (**4**)–(**14**) below on an orbital shaker, at room temperature unless otherwise indicated. Use wide-mouth Pasteur pipettes for transferring brains between solutions or change solutions in the same vial with incomplete draining.

4. Wash in TBS, 3 × 15 min.

5. 80% methanol / 20% DMSO, 2 h.

6. rehydrate in one of two ways: 1) rapid alternative: *directly into 0.1M Tris buffer, pH 7.4. 2)* gradual alternative: 100% methanol, 1 h.

• At this stage the brain can be stored indefinitely at –20°C. In this case, let come to room temperature before proceeding.

• 90%, 70%, 50%, 30% and 0% methanol in 0.1M Tris buffer, pH 7.4, 10 min each.

• Prepare dilutions well in advance, as mixing Tris buffer with methanol will drive air out of solution and bubbles may form in the tissue.

• Transfer brains onto Nunc 4-well plates (2 × 2 wells / plate; Nunc "Multidish" Cat. No. 176740, Thermo Fisher Scientific). During antibody incubations, seal the wells of the plates with adhesive film, e.g., Computype high-tack (acrylic based adhesive) polyester sealing film (Thermo Fisher Scientific). Wide (>1.5 cm) adhesive tape or insulating tape will also do. Steps (7 – 11) are on the 2 × 2 well plates:

7. 5% normal goat serum, 1% DMSO, 0.005% NaN_3 in 0.1 M PBS (PBSd-NGS), 1–2 h.

8. anti-SYNORF1 1:30 in PBSd-NGS, 3.5 days at 4°C.

9. Wash in PBSd for 3 × 2 h.

10. Cy2-conjugated goat anti-mouse antibody 1:100 in PBSd-NGS, 2.5 days at 4°C (add 0.5 µg/ml DAPI for a nuclear counterstain).

• Transfer back into ~10 ml capacity glass vials for steps (**11–14**):

11. 1%, 2%, 4%, 8%, 15%, 30%, 50%, 60%, 70%, 80% (1 h each) glycerol diluted in Tris buffer, with DMSO to 1% final concentration.

Preparations can be stored over night in the refrigerator at any stage.

OR:

• *wash 3 × 2 h in PBSd, then 30%, 50%, 70%, 90%, 96% Ethanol, 20 min each.*

• Ethanol, 3 × 30 min.

12. Underlay ethanol with methyl salicylate, wait for brain to sink (no agitation).

13. Aspirate fluid from top down, replace with fresh methyl salicylate for 30 min.

14. Mount in fresh methyl salicylate.

Equipment needed

• Access to confocal microscope equipped with laser lines for Cy2, Cy3 and DAPI, and with a 10´ dry lens, ideally with 0.4 numerical aperture and long working range.

• *Orbital shaker;* antibody incubations are on the shaker for several days at ~4°C, so shaker needs to go into a cold room (or, if no cold room available, into a refrigerator with the power cord through the shut door). Other incubations are on the shaker at room

temperature, so we would need to move the shaker in and out of the cold room unless we have two shakers (one cold, one room temperature) that can be shared with other people.

- Dissection stereomicroscope (with good epi-illumination!)
- Dissection instruments, including
- Razor blade breaker (micro-scalpel) and blades
- Dupont or equivalent watchmaker forceps, fine and coarse
- Iridectomy scissors
- Fine scissors
- Dissection dishes with wax or sylgard layer at bottom, ~ 5 cm diameter. Mix bee's wax or Sylgard with charcoal powder to give deep black background. Else clear plastic or glass Petri-dishes with clear Sylgard (can be used against black background).
- Minuten pins and normal pins.
- Glass Pasteur pipettes and fitting rubber "balloons"
- glass vials ~10 ml capacity (~2 cm diameter, ~3 cm tall) with plastic snap-on lids. "2 × 2" (4-well) plates: Nunc "Multidish" Cat. No. 176740, Thermo Fisher Scientific.
- Set of adjustable volume Pipettors, to cover 1000 µl down to 1 µl;
- Pipettor tips
- Eppendorf tubes 1.5 ml
- 15 ml and 50 ml "Falcon" style disposable plastic tubes if available; else 10 ml, 20 ml and 50 ml measuring cylinders and glass bottles;
- microscope slides
- metal washers, ~0.7 mm thick and ~1.5 mm thick, inner diameter (i.e., central hole) ~7 mm, outer diameter ~ 20 mm
- cover slips, round, ~ 12–15 mm diameter (large enough to easily cover central hole of washer)
- "Superglue" (cyanoacrylate glue)
- Wide (>2 cm) adhesive tape.
- 200 ml and 500 ml glass bottles for solutions.

Reagents

- For buffers and salines: Tris base (powder), HCl, NaOH, NaCl, mono- and di-basic sodium phosphate (NaH_2PO_4 and Na_2HPO_4, amount of crystal water does not matter), KCl, $CaCl_2$.

For zinc-formaldehyde fixative (Ott 2008 J. Neurosci. Methods):

- Zinc chloride ($ZnCl_2$)
- Formalin (commercial ~40% formaldehyde solution)
- Sucrose
- Dimethyl sulfoxide (DMSO)
- Methanol
- Phosphate-buffered saline
- Sodium azide (NaN_3)
- Triton X-100
- Normal goat serum (NGS; Sigma)
- 4′,6-diamidino-2-phenylindole (DAPI);
- used at 0.5 mug/ml from 0.5 mg/ml stock in water.
- Ethanol
- Glycerol
- Methyl salicylate
- Antibodies (I can provide these if necessary; but we need to think about how to best get them to the research station; fedex?)
- Monoclonal mouse anti-synapsin antibody 3C11 (anti-SYNORF1; Klagges et al., 1996), available from the Developmental Studies Hybridoma Bank (University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA).
- Some rabbit primary antibody that is guaranteed to work irrespective of species, for double-labelling; I was thinking of anti-serotonin from Sigma.
- Cy2-conjugated Goat anti-mouse: Jackson ImmunoResearch,
- Goat Anti-Mouse IgG (H+L) ML (min X Hu, Bov, Hrs, Rb, Sw Sr Prot), Cat.No. 115-225-146
- Cy3-conjugated Goat anti-rabbit: Jackson ImmunoResearch,
- Goat Anti-Rabbit IgG (H+L) ML (min X Hu, Ms, Rat Sr Prot), Cat.No. 111-165-144

Solutions

- Physiological Tris-buffered (not phosphate-buffered!!!) saline suitable for dissecting marine invertebrates
- Tris/HCl buffer (TB), 0.1 M, pH 7.4
- 0.2 M phosphate buffer stock (Na_2HPO_4 / NaH_2PO_4), pH 7.4
- Phosphate-buffered saline (PBS): 0.1 M sodium phosphate buffer, 140 mM NaCl, pH 7.4

• Zinc-formaldehyde fixative (Ott 2008 J. Neurosi. Methods); I'll mix this up myself as long as we have the ingredients.

• 90%, 70%, 50% and 30% methanol in 0.1 M Tris buffer (simply mix methanol and 0.1 M Tris buffer and 9+1, 7+3 etc., i.e., the 0.1 M refers to molarity before mixing, not final).

PROTOCOLS OF NATALIA M. BISEROVA

HOW TO PREPARE WORMS FOR CONFOCAL MICROSCOPY? (IMMUNOCYTOCHEMICAL TECHNIQUES FOR CESTODA)

General comments

Laser scanning confocal microscopy represents one of the most significant advances in optical microscopy ever developed, primarily because the technique enables visualization deep within both living and fixed cells and tissues and affords the ability to collect sharply defined optical sections from which three-dimensional renderings can be created

Confocal microscopy offers several advantages over conventional widefield optical microscopy, including the ability to control depth of field, elimination or reduction of background information away from the focal plane (that leads to image degradation), and the capability to collect serial **optical sections** from thick specimens. The basic key to the confocal approach is the use of spatial filtering techniques to eliminate out-of-focus light or glare in specimens whose thickness exceeds the immediate plane of focus. There has been a tremendous explosion in the popularity of confocal microscopy in recent years, due in part to the relative ease with which extremely high-quality images can be obtained from specimens prepared for conventional fluorescence microscopy, and the growing number of applications in cell biology that rely on imaging both fixed and living cells and tissues. In fact, confocal technology is proving to be one of the most important advances ever achieved in optical microscopy.

Biological laser scanning confocal microscopy relies heavily on fluorescence as an imaging mode, primarily due to the high degree of sensitivity afforded by the technique coupled with the ability to specifically target structural components and dynamic processes in chemically fixed as well as living cells and tissues.

Fluorescence is the property of some atoms and molecules to absorb light at a particular wavelength and to subsequently emit light of longer wavelength after a brief interval, termed the fluorescence lifetime. Fluorophores chosen for confocal applications must exhibit a brightness level and signal persistence sufficient for the instrument to obtain image data that does not suffer from excessive photobleaching artifacts and low signal-to-noise ratios.

There are direct and indirect methods for staining of preps for confocal microscopy. Good results can be obtained with chemical fluorochrome dyes, like DAPI or Phalloidini TRITC or can be used immunostaining with primary antibodies conjugated with fluoro-

chrome or two steps staining with primary and secondary antibodies, well known “the sandwich method”.

Fixation

For whole-mounts cestodes, it usually needs skinning in dist. H_2O before fixation

Stefanini’ fixation:

- 2% paraformaldehyde and 15% picric acid (15 ml saturated picric acid in 100 ml of fixative solution) in 0.1 M sodium-phosphate buffer saline [PBS] pH 7.4; 4ºC.

The most common fixative solution:

- 4% paraform in 0,1M PBS, pH 7.4; 4ºC

The optimal time of fixation correlate with a size of the tissue pieces, and it is between 1- 4h and 2-3 days in the fixative solution in the refrigerator (4ºC). Longer time of the fixation can decrease the immunoreactivity of the tissue.

Comments:

It is essential to keep antigens accessible in the tissue. If you need to store the material for a long time before you are able to develop immunoreactions, there are two possibilities to keep materials.

After fixation and washing in 0.1M PBS (3 × 5):

- transfer to buffer with 0.03% sodium azide and store in refrigerator (4ºC).
- dehydrate to 70% ethanol and keep frozen (-20ºC) [except for Phalloidin’ staining!]

If you plan to make cryosections on the cryotome, after washing in buffer it is possible to store material in 10% sucrose in the same buffer.

Proceeding

Stock solutions:

- Primary antibodies
- Secondary antibodies conjugated with fluorophores
- 0,01M PBS ph 7.4 with 0.03% sodium azide
- Tryton-X100, detergent, for an easy penetration of the antibodies.
- For suppression of the unspecific reactions:
- BSA - bovine serum albumin
- NGS – normal goat serum

Immunostaining:

- washing in 0.01M PBS (6×10 min),
- preincubation in 1-10% NGS (or in BSA) in 0.01M PBS containing 2–5% Triton X-100

• incubation in primary antiserum diluted in 0.01M PBS containing 2–5% Triton X-100 and 1-10% NGS from 24 h to several days at 4°C in shaker.

• washing in 0.01M PBS containing 1% Triton (6×10 min) in shaker;

• incubation with the secondary antibodies conjugated with fluorophores diluted in 0.01M PBS containing 1% Triton and 10% NGS from 1–12 h at 4°C; in shaker.

• washing in 0.01M PBS containing 1% Triton (6×10 min)

• whole-mounts keep in 50% glycerol in 0.01M PBS, at 4°C (or -20°C)

Comments:

Whole-mounts cestodes as usual have a large size and need a long time for incubation. For best result you can use a special shaker.

Another possibility is to make the cryosections:

• Worms are embedded in Tissue Tek and cut at 15 μm with an ultracryostat.

• The sections are collected on gelatine-coated slides and keep frozen.

• Immunostaining of sections develops on slides on which solutions are dropped.

IMMUNOGOLDSTAINING, OR HOW WE HAVE TO PREPARE WORMS FOR TEM IMMUNOLABELING?

General comments

Ultrastructural researches give us detailed morphology of cells and tissues. In the same time we often need to know some physiological or biochemical parameters, like mediator or protein specificity in investigating cells. Tissue is usually prepared for electron microscopic immunocytochemistry in order to learn something about the ultrastructural location of the antigen or the fine structure of the cells that contain the antigen. Tissue can be prepared for for electron microscopic immunocytochemistry by pre-embedding or post-embedding techniques. If you want to know vesicles contents or chemical specificity of neurons, or to label protein fibers, you can use post-embedding immunogoldstaining of ultrathin sections.

Fixation

• Size of the tissue pieces must be small, about 1 - 2 mm^3

• 2% (up 4%) paraform + 1% (0.05-0.5%) glutaraldehide in 0,1M PBS, pH 7.4, 4°C (from 4h to 3 days)

Proceeding

• wash in 0.1M PBS, 2 × 10 min

• postfixation in 1% OsO_4 in 0,1M PBS, pH 7.4, (from 30 - 60 min)

• 2% uranyl acetate in dist. water (30 - 60 min, this step is not obligate and used for

- the contrasting of membranes)
- Dehydration in alcohol 30%, 50%, 70% - 2 × 15 min; 96%, 100% - 2 × 20 min

Embedding

- LRWhite resin 2 × 12h at the room temperature
- For hard tissue or large pieces you can use next protocol of embedding:
- LR-White+96% ethanol 1:1, 1h in rotor;
- LR-White+96% ethanol 3: 1, 5h in rotor;
- LR-White, 12h in rotor + 12h in rotor;
- LR-White in gelatin capsule in 60°C, 24h
- Polymerization (hardening)

It is essential, that hardening (24h at 60°C) must take place in the absence of air. The capsules, therefore, have to be covered with its caps to exclude air!

Comments:

Different mediums (resins) have different protocols:

- Lowikryl needs the UV and low temperature (-35°C) for embedding and polymerization;
- Durcupan and Spurr have law viscosity and short time for embedding and hardening.

Immunostaining on thin (50-80nm) sections

For immunogoldstaining, thin sections must be mounted on the gold, or nickel slots or grids.

Stock solutions:

- Tris 0.1M pH 7.4
- 200 ml H_2O
- 2,42 g Tris
- 1.8 g NaCl - adjust to pH 7.4
- Tris 0.1M pH 8.2
- 200 ml H_2O
- 2,42 g Tris
- 1.8 g NaCl - adjust to 8.2
- Tris 0.1M pH 7.4 + 1% NGS
- Tris 0.1M pH 7.4 + 5% NGS

Comments:

Instead of NGS it is possible to use BSA (bovine serum albumin) and also PBS buffer is very useful for immunogold methods.

Proceeding

As usual all steps proceed on the drops in a moist chamber. You can use Petri dishes with moist paper covered by parafilm or special chamber.

1. Etching

- Sodium metaperiodate ($NaIO_4$) saturated at 50°C (etching of the embedded medium), 5 min

2. Washing

- H_2O dist., 5 × 1 min

3. Preincubation

- 0.1M Tris buffer, pH 7.4 +5% NGS (normal goat serum), 30 min

4. Incubation

- Primary antibodies, diluted in 0.1M Tris, pH 7.4 containing 1% NGS, 4°C, overnight

5. Washing

- H_2O dist., 8 × 5 min

6. Changing pH

- 0.1M Tris, pH 8.2, 5 min

7. Conjugation

- Secondary antibodies, conjugated with 10 (12,15) nm colloidal gold
- dilution 1:30 in 0.1M Tris, pH8.2, 1 hour, 4°C

8. Washing in buffer

- 0.1M Tris, pH 8.2, 5 × 4 min

9. Washing

- H_2O dist., 8 × 5 min

10. Contrasting membranes

- Staining with 0.4% lead citrate
- (or double-staining with 4% uranyl acetate in dist. water and 0.4% lead citrate).

Comments:

The immunostaining of a material, embedded in Durcupan ACM, it is possible to proceed without etching. Firstly, drops of PBS with 0.5% BSA –10-20 min (without etching); then incubation with the antisera, washing 7-8 times; incubation with gold15-labeled secondary antibodies; washing with PBS; double-staining with uranyl acetat and lead citrate.

Control

It is very important to have control staining for verification of immunoreaction!

For this case, instead of primary antibodies (4th step) you have to put slots in the same buffer solution without primary antibodies. Other steps are the same.

For references:

Biserova N.M., Gustafsson M.K.S., Reuter M., Terenina N.B. 1996. The nervous system of the pike-tapeworm *Triaenophorus nodulosus* (Cestoda: Pseudophyllidea) - ultrastructure and immunocitochemical mapping aminergic and peptidergic elements. Invertebrate Biology. V.115. N 4. P.273-285.

Biserova N.M., Dudicheva V.A., Terenina N.B., Reuter M., Halton D.W., Maule A.G., Johnston R., Gustafsson M.K.S. 2000. The nervous system of *Amphilina foliacea* (Platyhelminthes, Amphilinidea). An immunocytochemical, ultrastructural and spectrofluoromentrical study. Parasitology, 121, p.441-453.

Skiebe P, Biserova NM, Vedenina V, Borner J, Pflueger H-J. 2006. Allatostatin-like immunoreactivity in the abdomen of the locust *Schistocerca gregaria*. Cell and Tissue Res. 325: 163-174

Biserova N.M. 2008. Do glial cells exist in the nervous system of parasitic and free-living flatworms? An ultrastructural and immunocytochemical investigation. Acta Biologica Hungarica. V.60. (Suppl.30). P. 208-219

PROTOCOL OF DR. TH. STACH

Material

- 5 beaker (1L)
- 5 tanks 30-50 L
- 1 redlight
- 1 L citricacid (20%)
- glass pipets
- 1 blue light
- 200 um mesh
- petri dishes
- dark room
- collecting vials (5 ml)
- TEM-fixatives:Sorensensbuffer (pH 7.2,Osm. 0.2, 1L), sodium-cacodylatebuffer(pH 7.2, Osm.0.2, 1L), glutaraldehyde (25%,10 × 10 ml), OsO_4 (4%)
- $MgCl_2$ (7%, 1L)
- Bouin's fixative(10 × 10 ml)

PROTOCOL OF DR. L. P. NEZLIN

- Preparation of solutions: buffer, fixative, blocking solution, etc.
- Dissection and fixation of specimens.
- Multicolor immunostaining.
- Imaging in epifluorescence and laser scanning confocal microscope.
- Image processing and analysis.

List of chemicals

- Paraformaldehyde
- Na_2HPO_4
- NaH_2PO_4
- Bidistilled water
- Normal goat serum
- Triton X100
- Glycerol
- Anti-serotonin antibodies, developed in rabbit
- Anti-tubulin antibodies, developed in mouse
- Goat-anti-rabbit IgG labeled with Alexa-488
- Goat-anti-mouse IgG labeled with Alexa-546
- DAPI

List of necessary equipment

- Scales 0.001 – 100 g
- pH-meter
- microscope glass slides
- coverslips
- forceps
- Pasteur pipettes
- glass vials
- stereomicroscope

BACKFILLING A NERVE WITH BIOCYTIN OR DEXTRAN

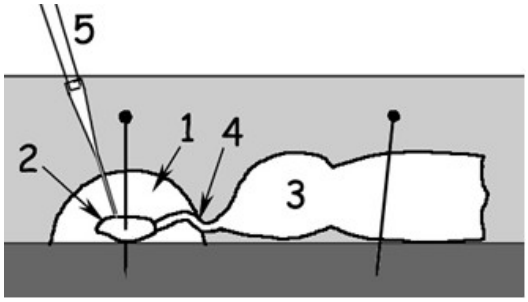
Leonid Nezlin

Below, the method of backfilling nerves is described. The following tracers can be used: biocytin, neurobiotin, dextran conjugated with a fluorescence label (dextran must be anionic).

1. Dissolve 0.5 – 1% biocytin in distilled water (must be fresh! so make not more than 100 ml – enough for 10 – 20 preparations).

2. Anesthetize the animal; dissect the brain with the nerves of interest in physiological saline (PS) or sea water.

3. Fill a Petri dish (silicone covered bottom) with PS (Fig. 1), put a drop of paraffin or vaseline oil (1) and pin inside a piece of filter paper (2) presoaked with PS. Pin the brain nearby (3), attach the nerve (4) to the paper with the help of thin forceps and add several microliters of biocytin or dextran solution to the paper with the help of a pipette with a long thin tip (5). Important! Avoid leakage of biocytin from inside the oil drop. To control it visually, the biocytin solution can be colored with a vital dye (i.e., neutral red).



4. After 2 – 4 hours at room temperature, detach the brain, put them in fresh PS and leave at 10°C for 2 – 4 hours more.

5. Fix the brain in 4% formaldehyde in 0.1 M phosphate buffer (PB) for 3 – 10 h.

6. Wash in PB. If the brain is processed as a wholemount go to step 11.

7. If sectioning is necessary, immerse the brain in 30% sucrose in PB for 3 – 5 hours for cryoprotection (the brain is ready for sectioning when it sinks to the bottom.)

8. Section the brain in a cryostat. Collect the slices on chrom-alum-gelatin coated glass slides*.

9. Air-dry the slides for 15 – 20 min.

(The brains can be also sectioned in a vibratome and further processed free-floating).

10. Wash the slides in PB, 3 × 15 min.

11. Apply avidin or streptavidin conjugated with a fluorescent label (1:200 – 1:400 in PB) for 5 – 10 hours at room temperature, in darkness.

12. Wash in PB, 3 × 5 min.

13. Embed in PB-glycerol (1:2). Coverslip, examine.

In case of dextran, steps 11 and 12 are omitted.

Solutions:

Stock solution for dextran

1% DMSO, 10 mM NaCl, 5 mM HEPES, in dH_2O, pH 7.2 – 7.5

Phosphate buffer (PB) 0.2 M

Dissolve 22.94 g Na_2HPO_4 and 4.6 g NaH_2PO_4 in 800 – 900 ml dH_2O, adjust pH to 7.2 – 7.4, bring the volume to 1000 ml.

Phosphate buffer (PB) 0.1 M

Dilute 0.2 M PB 1:1 with dH_2O

Fixative

Mix 50% of 0.2 M PB, 4% of formaldehyde powder and 46% of dist. water. Warm on a magnetic stirrer up to 60 – 70°C (do not boil!) until the solution becomes clear, cool down and store in a fridge.

*** Chrom-alum-gelatin**

(a) Add 1 g of gelatine to 150 ml of hot dist. water, wait until it dissolves. (b) Dissolve 0.1 g of chrom-alum in 50 ml of dist. water. Mix (a) and (b). Store in a fridge.

To coat glass slides:

1. Load slides in a cassette, immerse in a detergent solution for 10 – 15 min.
2. Wash in tap water for 20 – 60 min.
3. Rinse in 2 – 3 changes of dist. water.
4. Warm the solution of chrom-alum-gelatin up to 25 – 30°C.
5. Immerse the slides into it for 5 min.
6. Put the cassette on a wipe for several minutes and dry at 50 – 60°C.

PROTOCOLS OF PROF. A. WANNINGER

ICC PROTOCOL FOR NEUROTRANSMITTER STAINING OF LOPHOTROCHOZOAN LARVAE

11. *Fix in 4% PFA (in 0.1M PBS), 12 hrs (o/n) at 4°C; or 4 hrs at RT (probably better). NB: Don`t fix longer than 20 hrs.*

NB: For anti-Tyrosine Hydroxylase (TH) and anti-dopamine beta hydroxylase (DβH) fix in cold 100% methanol at -20°C for 10 – 30 min. (Dickinson & Croll 2003)!!

12. *Wash in 0.1M PBS+0.2-10 % Triton X-100+0.1% NaN_3 (=PTA); 15-60 min., RT, 3 changes, or, if specimens should be stored, wash in 0.1M PBS+0.1% NaN3 and store in this solution (up to 1 yr at 4°C).*

13. *If necessary: decalcify in 2% EDTA or 0.05M EGTA; 1-2 hrs at RT should be enough;*

14. *If decalcified, wash again as under 2).*

15. If stored in PBS+0.1% NaN_3: Wash in 0.1M PBS+0.2-10% Triton X-100+0.1% NaN_3 (=PTA); 60 min.

16. Blocking of unspecific binding sites: 6% normal goat serum (NGS) in PTA (=Block-PTA); (o/n at 4°C).

17. Primary antibody (pAB): dilute pAB in BlockPTA. Concentrations we use:

Serotonin: 1:800
FMRFamide: 1:400
α-Tubulin: 1:500 (1:100 - 1:300 for cirripedes)
Histamine: 1:1000

7A) For double labelling with ABs from DIFFERENT hosts:

Make cocktail of the 2 primary ABs in the respective working conc. (e.g. anti-Serotonin from rabbit and anti- α-Tubulin from mouse) and incubate.

7B) For double labelling with ABs from SAME host:

Use one of the 2 desired antibodies in the respective working concentration

Incubation: ~24hrs at 4°C (maybe considerably longer for cirripedes).

18. Wash in BlockPTA; 6-12 hrs or over night at 4°C, 4 changes.

19. Secondary antibody (sAB) (conjugated with fluorescent dye):

dilute sAB in BlockPTA:

anti-rabbit Alexa Fluor 568 or 633 1:100 (maybe better 1:300)
anti-mouse Alexa Fluor 488 or 568 1:100 (maybe better 1:300)
anti-mouse FITC: 1:300 (1:100 for cirripedes)

9A) For double labelling with ABs from DIFFERENT hosts:

Make cocktail of the 2 secondary ABs in the respective working conc. (e.g. anti-mouse Alexa Fluor 488 **and anti-rabbit** Alexa Fluor 633**) and incubate. Make sure the 2 ABs are conjugated to a different fluorescent dye (e.g., AlexaFluor 488 and 633)!!**

9B) For double labelling with ABs from SAME host:

Use a matching secondary antibody in the respective working concentration

Incubate o/n or 24h at 4°C (maybe considerably longer for cirripedes)**.**

20. Wash in PBS **without NaN_3** (NaN_3 quenches fluorescence!!); 6-20 hrs (o/n) at 4°C (1-4h if clearing follows), 4 changes.

10B) For double labelling with ABs from SAME host:

Wash in BlockPTA; 6-12 hrs or over night at 4°C, 4 changes.

Use the second desired primary antibody in the respective working concentration

Incubate o/n or 24h at 4°C (maybe considerably longer for cirripedes).

Wash in BlockPTA; 6-12 hrs or over night at 4°C, 4 changes.

Use the matching second secondary antibody in the respective working conc.

Incubate o/n or 24h at 4°C (maybe considerably longer for cirripedes).

Wash in PBS without NaN3 (NaN3 quenches fluorescence!!); 6-20 hrs (o/n) at 4°C (1-4h if clearing follows), 4 changes.

21. If staining of cell nuclei is desired: add 1 or a few drops of DAPI or a working dilution of 10µl/200µl PBS of a 100µg/ml stock solution HOECHST 33258 to sample.

Incubate for 5-30 min. at RT.

22. Wash in PBS without NaN_3 (3 × 15 min at RT).

23. Mount in Vectashield or in 70% Glycerol or Fluoromount G

OR: if clearing of tissues is required:

After step 10 or 11 or 12):

24. dehydration in ETOH series (each step 5-10 mins):

A.dest. - ETOH 30% - 50% - 70% - 80% - 90% - 3 × 100

25. clearing:

- make solution of benzyl benzoate:benzyl alcohol = 2:1 (BBA)
- make solution of BBA:ETOH = 1:1 (BBA-ETOH)
- transfer embryos/larvae into BBA-ETOH (10 mins)
- transfer embryos/larvae into BBA. This should make your specimens transparent enough for a good analysis

ALTERNATIVE: transfer specimens in 100% ETOH on slide, remove ETOH, add few drops of BBA and add cover slip.

26. store at 4°C or –20°C.

ACTIN STAINING USING FLUORESCENCE-COUPLED PHALLOIDIN

Before you start:

- Dissolve the fluorescence stain (usually comes as a powder; e.g., ALEXA 633 phalloidin) according to manufacturer's advice and store at -20ºC
- Use this stock solution for all experiments

STAINING PROCEDURE:
(use PFA fixed material stored in PBS)

- Permeabilisation: in PBT (0.1 M PBS + 0.2 – 10% Triton X-100)

60 min – overnight

Staining procedure:

(NB: all steps in the dark!)

Use a 1:40 dilution of the phalloidin/phallacidin stock solution in PBT:

- take 5 µl of the stock solution and pipet it into an embryo dish.
- Let stand in the dark for about 10 min until the methanol has evaporated.
- Pipet the larvae together with 195 µl PBS-T into the embryo dish containing the phalloidin

60 min – overnight

If counterstaining of cell nuclei is required:

- add 1 µg/ml DAPI to the sample and incubate together with phalloidin
- Wash in 0.1 M PBS

3 × 15 min

Embed on glass slides in Vectashield or 70% Glycerol or Fluoromount G (if the latter is used store at least o/n in the fridge prior to analysis)

Store at 4ºC in the dark (for long-time storage store in freezer at -20 ºC)

REARING, RELAXATION, FIXATION, DECALCIFICATION OF SMALL MARINE INVERTS INCL. LARVAE

update AW 16.03.2007

I. REARING OF LARVAE

Mix of antibiotics

In order to prevent fungal or bacterial infection of your larval cultures it is ofeen useful to add a mix of antibiotics/fungicids to the cultures

OBS! the following:

(1) It is recommended to NOT add antibiotic/fungicid substances to early embryonic (cleavage) stages because that can lead to abnormal development (generally avoid to overdose – antibiotics are in fact toxic if used in high concentrations). Only add them to cultures with already swimming larvae. Generally, it is best to try without antibiotics and only use them if the cultures crash.

(2) Remember that, once in solution, the antibiotics only stay active for 2 days at the most (but the seawater should be changed once daily anyhow in most cases).

The mix we normally use is:

Penicillin G	1.2 g
Streptomycin (-sulfate)	1 g

(store at 4 ºC)

Dissolve in 200 ml Millipore filtered seawater (MFSW);
make 20 × 10 ml aliquots and freeze at -20ºC;
use 1 aliquot for 1 litre MFSW (i.e., final working concentration is 60 mg penicillin G and 50 mg streptomycin (sulfate) per litre MFSW).

II. RELAXATION

Magnesium chloride

(250 ml; 7.14 %; for relaxation)

($MgCl_2$)	(17.85 g)
$MgCl_2 \times 6H_2O$	38.097 g

fill up with distilled water to a total volume of 250 ml;
should be applied in a 1:1 dilution in SW;

NOTE: sometimes it can be of advantage if the $MgCl_2$ concentration is subsequently raised to a final conc. of 7.14% (i.e., all the SW has been washed out) – in particular, if the specimens have strongly developed retractor muscles.

III. FIXATION

1. TEM and SEM fixatives

TEM fixation according to Eisenmann & Alfert 1982 (J. Microsc. 125: 117-120)

Fixatives:

a) Glutaraldehyde fixative = primary fixative ("Glu-fix"):

4% glutardialdehyde in 0.2 M sodium cacodylate buffer (0.1 M NaCl, 0.35 M sucrose) pH: 7.2

Buffer I:

sodium cacodylate	2.14 g
NaCl	0.292 g
sucrose	5.99 g

fill up with distilled water to total volume of 50 ml
adjust pH to 7.2 (with HCl)

Glu-fix:

25 % glutaraldehyde	2 ml
buffer I	10.5 ml

b) Osmiumfixative = post-fixative ("Post-fix"):

1% OsO4 in 0.2 M sodium cacodylate buffer with 0.3 M NaCl; pH = 7.2

Buffer II:

sodium cacodylate	1.712 g	**3.424**
NaCl	0.856 g	**1.712**
	fill up with A. dest. to a volume of 20 ml	fill up with A. dest. to a volume of 40 ml

adjust pH to 7.2 (using HCl)

Osmiumfixative:

4% osmiumtetroxyde	1 part	2 ml	**4 ml**
A. dest.	1 part	2 ml	**4 ml**
buffer II	2 parts	4 ml	**8 ml**

check if pH remains at 7.2

(**Alternative: fixation cocktail**:

Glu-fix	9.5 ml
Post-fix	0.5 ml

NB: do only make up IMMEDIATELY prior to usage!!

Molekular weights:

Na-cacodylate: MW = 214.13

Sucrose: MW = 342.30

NaCl: MW = 58.44

ALL fixations and washes at 4°C

fix in Glu-fix.: 1-2 h

wash in buffer I + A. bidest 1:1: 3 × 15 min

fix in Post-fix: 1-2 h

If cocktail used:

fix 10-15 min

wash in buffer II + A. bidest 1:1: 3 × 15 min

NB: for SEM fixations, the specimens can be stored in the 4% GLU fix; do the post-fixation and all further procedures back home

Normally, standard PFA fixed specimens (see below) are good enough for SEM analysis

If delicate calcareous structures need to be preserved (e.g., larval shells), try a simple (and deadly poisonous!!) solution of 1% OsO_4 in A. dest. for 1 h. After that, was the specimens in A. dest and go through a graded ETOH series and store in 70% ETOH. Do all further dehydration, critical point drying, etc. back home. Tissue preservence is pretty poor using only OsO_4 fix and should thus only be used for SEM.

2. ICC buffers and fixatives

5x phosphate buffer (PBS) for ICC

(Sörensen 1909, Z. Biochemie 22: 352);

500 ml; pH: 7.2-7.4; 5x stock solution

(Na_2HPO_4) $Na_2HPO_4.2H_2O$	(26.7 g) 33.48 g
(NaH_2PO_4) $NaH_2PO_4.H_2O$	(6.9 g) 7.93 g

- fill up with A.dest to a total volume of 500 ml; check/adjust pH to 7.2-7.4. This is your 5x concentrated stock solution; store at RT
- make a 1:5 dilution with A. dest. to yield 0.1M working solution; check pH!!
- for use as wash/storage medium add 0.1% NaN_3 (sodium azide) (=0.1g/100ml)

Immunohistochemical blocking buffer (dried milk based) (MB)

(Fabian & Seyfarth 1997; modified by Tim Wollesen)

- applied on larvae, juveniles, and adults of the gastropod Aplysia
- instead of conventional PBS to reduce background staining
- preincubation (if necessary) and 1. AB with NGS and MB

Protocol:

NaCl	85 mg
BSA (bovine serum albumin)	25 mg
MP (fat-free dried milk powder)	300 mg

- dissolve in 10 ml phosphate-buffer and stir for 10 min
- centrifuge for 5 – 10 min to clear the solution
- filtrate the solution

Application:

- For the primary antibody preparation use the blocking buffer supplement with 40 µm/ml (4%) NGS (normal goat serum) to dilute antibodies. If the primary antibody is an unconcentrated serum dilution and further dilution is unacceptable, preincubate the preparations for 1-2h at RT/over night at 4°C in the MB without the antibodies. In a second step do the incubation with the undiluted antibody.
- Rinse samples thrice in conventional PBS for more than 10 minutes each.
- The secondary antibody is diluted in PBS.
- Rinse samples thrice in PBS for more than 10 minutes each.

Paraformaldehyde fixative (PFA) (standard fix we use for ALL ICC studies and also as a standard SEM fix)

(Rieger et al. 1994); (4% in 0.1 M PBS; 500 ml)

Paraformaldehyde	20 g

- **OBS! Work under fumehood!!**
- add approx. 300-400 ml 0.1M PBS. Put on heating plate with magnetic stirrer and warm up (up to max. 80°C; take care and don't boil!). Add carefully a few drops of NaOH to the solution until all PFA has been dissolved. Fill up with 0.1M PBS to a total volume of 500 ml.
- Adjust pH to 7.2-7.4
- **NB**: some people add 10% (= 50g) sucrose to the fix to adjust its osmolarity to SW, but we usually don't bother...

Stefanini – Fixative (traditional fix, we normally don't use it anymore)

(Stefanini, 1967?, Fichtner, 1994)

Dissolve 20 g Paraformaldehyd in 150 ml saturated picric acid (freshly made; dissolve picric acid in warm/hot A. dest.) at approx. 80°C on magnetic stirrer. Add drops of NaOH to aid dissolving. Wait until solution has cooled down to RT and fill up to 1000 ml with 1x PBS. Add 100g sucrose and adjust pH to 7.2-7.4.

IV. DECALCIFICATION

The best and most efficient decalcification medium is a 50 mM (0.05 M) solution of EGTA; a milder one that takes MUCH longer is EDTA; therefore, we normally use EGTA:

EGTA:

(100 ml of a 50 mM solution in A. dest for decalcification)

Dissolve 1.9 g EGTA in ca. 80 ml A. dest. at approx. 60-80°C and, if required, with the addition of drops of NaOH. Once solution has cooled down, fill up to 100ml and adjust pH to 7.2-7.4.

EDTA:

(2% for decalcification in A. dest; Romeis, 1989)

Titriplex III	10 g

Dissolve in approx. 200ml at max. 80°C; add drops of NaOH if required. Let cool down, fill up to 500 ml and adjust pH to 7.2-7.4

PROTOCOL OF DR. M. PLYUSCHEVA

VISUALIZATION OF NERVE SYSTEM, INVOLVED IN BIOLUMINESCENCE REACTION OF SCALE-WORM *HARMOTHOE IMBRICATA* (POLYCHAETA, POLYNOIDAE)

Fixation

4% Paraformaldehyde on PBS 4 – 6 hours

Wash

PBS 3 times 30 minutes

Blocking

6% Normal Goat Serum on PBT (Triton X-100 5%)

4 hours – overnight

Wash

PBT 3 times 30 minutes

Primary antibodies

FRMF-amide (rabbit)

5-HT serotonin (rabbit)

ChAt4b1 (drosophila choline acetyltransferase fusion protein) (mouse)

α-tubulin (mouse)

Final dilution 1:800 (1,25 µl per 200 µl of PBT)

8 hours – overnight

Wash

PBT 3 times 30 minutes

Secondary antibodies

Alexa 488 anti-rabbit

Alexa 635 anti-mouse

Final dilution 1:800 (1,25 µl per 200 µl of PBT)

8 hours – overnight

Wash

PBT 3 times 30 minutes

During the first wash add 1 µl of DAPI per 200 µL of PBT to stain nucleus

Selected results of students experiments

1 – 5). Immunocytochemical (ICC) staining of the planktonic whole mounted animals. Colors of the dyes used: Red – anti-α-tubulin mouse. Green – anti-FMRF-amide rabbit. Blue – DAPI, nuclear dye. LSM Nikon A1. K. Kuznetsova, A. Makarova, S. Vodopyanov

6). ICC staining of the whole-mounted head of *Travisia forbesii* (Annelida, Polychaeta) with synapsin and serotonin (5-HT). Colors of the dyes used: Green – anti-synapsin mouse; Yellow – anti-5HT rabbit. LSM Nikon A1. S. Vodopyanov.