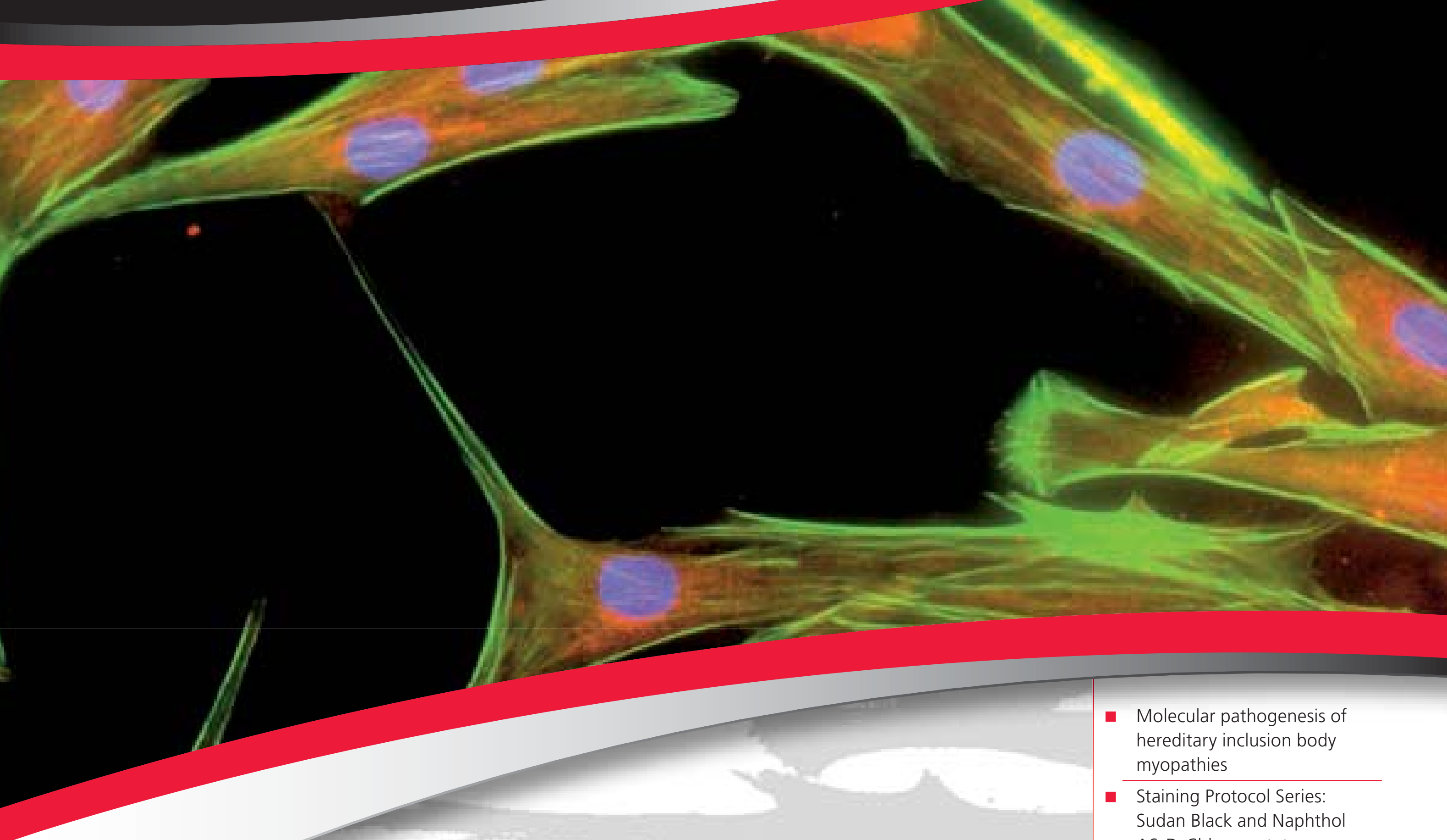
SIGMA Life Science

IN VITRO

Issue 1 • 2008

Academic and industry:

Interview with Ch. Thirion – Scientist and co-founder of SIRION biotech



- Molecular pathogenesis of hereditary inclusion body myopathies
- Staining Protocol Series: Sudan Black and Naphthol AS-D-Chloroacetate Esterase 90
- Atto labels and their conjugates – versatile, bright and stable tools for imaging
- Pseudomonas Media and Tests
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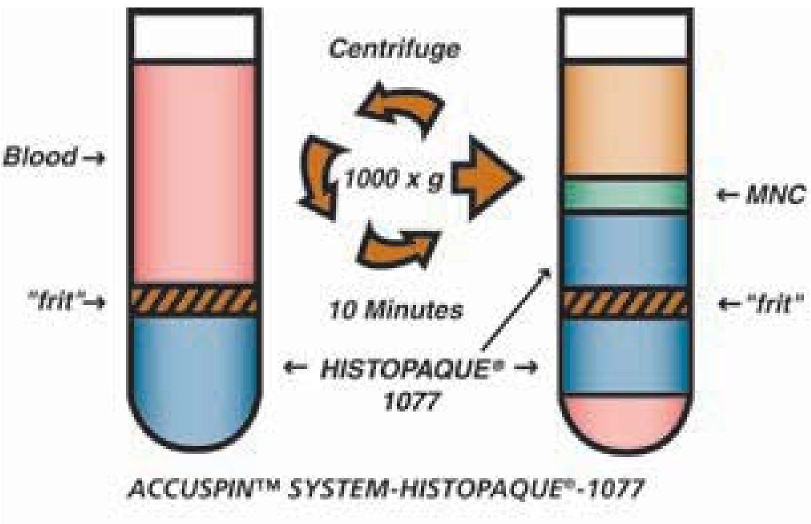

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Dear Colleague,

Sigma-Aldrich Chemie GmbH
Walter Gmelin, PhD

Welcome to the premier edition of IN VITRO, Sigma-Aldrich's newsletter devoted to histology and hematology.

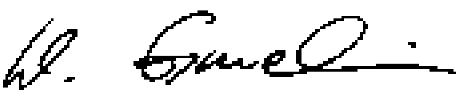
Sigma-Aldrich produces a variety of newsletters that focus on different fields of science, including biotechnology, analytical chemistry, organic synthesis and others. With IN VITRO, however, we have sought to take a slightly different approach by putting the spotlight on our customers and making their research the central theme. Each issue of IN VITRO will feature an article and an interview with one of our customers some fundamental aspect of their work. By tapping into our customer base for content, we hope to make IN VITRO an interesting read, and also give a taste of the wonderful diversity of their research and the multifarious nature of the scientific disciplines we serve.

While we're on the subject of diversity, the histology and hematology techniques themselves are used widely by scientists involved in biological, physiological, medical, pharmaceutical, clinical and biochemical research. Histology is the study of thin slices of tissue to observe its morphological and chemical characteristics, typically by using specific stains and dyes. Histopathology is histology applied to the diagnosis and treatment of diseases, like cancer, and, as this issue's feature article describes, muscular dystrophy and other neuromuscular disorders.

Muscular dystrophy (MD) is an indiscriminate disease that often strikes during the patient's youth and prime years of life. There are many different forms of MD and each has a specific set of symptoms, inheritance and causative agents. In the work featured in this issue of IN VITRO, PhD Christian Thirion of the Ludwig-Maximilians-Universität Munich and colleagues from Charité-Universitätsmedizin Berlin, Hadassah-Hebrew University Medical Center, University of Hamburg and Newcastle University have been studying a specific type of MD, inclusion body myopathy (IBM). Using hematoxylin and eosin staining and double immunofluorescence detection techniques, Dr. Thirion and his collaborators observed abnormalities in the arrangement of actin molecules in the muscle cells of patients with IBM. By linking these abnormalities to the expression of a muscle-specific p97/VCP gene, their work has implications in the inheritance and early detection of this form of MD, as well as increasing the understanding of the underlying physiological and molecular mechanisms.

In addition to the summary of Thirion's work on MD and the transcript of our interview with him, this issue of IN VITRO also contains articles on our products and services for histology and hematology researchers. We sincerely hope you find the content interesting and useful. Please look to future issues of IN VITRO where we will spotlight the work of other customers in the fields of histology or hematology.

Kind regards,



Walter Gmelin, PhD

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Interview with Dr. Christian Thirion, biochemist and co-founder of SIRION GmbH

SIRION GmbH was founded in December 2005 by biochemists Christian Thirion and Lars Behrend in the Munich biotech region. Since then, they have introduced innovative tools and services for RNAi research in academic and pharmaceutical settings. Uncharacteristic to their industry, SIRION has focused from the start on generating revenue from their products and services and, as a result, have been able to self-finance their ongoing R&D efforts. Since September 2007 SIRION has been operationally active and currently employs eight full-time scientists. The following is a transcript of an interview we conducted with Thirion in May 2008.

IN VITRO: A little over two years ago, you and a colleague started SIRION. How have you been able to keep new ideas flowing to maintain its growth and commercial viability?

Christian Thirion, PhD: In the past few decades, some fundamental enabling technologies have been invented, such as monoclonal antibodies and RNA interference. The potential of both of these techniques has not been completely explored. In the case of RNAi, indeed, we are just at the very beginning of our understanding. The course of development of both technologies has provided a fount of ideas for us that we have turned into products and services.

IN VITRO: In addition to running the company, you also hold an academic position at the University of Munich. How do you leverage your academic research to generate ideas for new products and services to offer through your company?

CT: The stimulating environment of collaboration found in an academic setting is invaluable. Our research group works at the cutting-edge of science in collaboration with other key scientists and research groups in our areas of interest. This collaboration is essential for knowledge and technology transfer. It not only facilitates our academic research, it also allows our company to stay at the forefront of science in order to maintain its technical relevance. On the other hand, the public funding behind most academic research puts more demand on generating sustainable research.

IN VITRO: Have you found working in these two very different environments beneficial?

CT: By looking at my academic research through the lens of an entrepreneur, I am able to see things from a different perspective. And it is possible to work successfully in both areas. Another example is Professor Ralf Wagner, who still works as professor while at the same time successfully running GENEART AG as a board member. The open exchange of concepts and ideas helps academic and commercial interests increase creativity and improve efficiency.

IN VITRO: Please describe SIRION's products and services for our readers.

CT: For molecular biologists and other scientists studying gene expression or regulation, SIRION offers a new and unique product, Q-tech, which comprises ready-to-use transfected cells with a pre-set gene knockdown. The customer combines the gene of interest for knockdown with the desired cell type and receives from SIRION the cell system with the knockdown. Our core competency in adenoviral transfection technology allows us to offer this unique and valuable service.

IN VITRO: What makes your service unique?

CT: Q-Tech overcomes several practical limitations of current RNAi technology and enables the researcher and developer to do fast, reproducible experiments with consistent quality of the source material. It is possible to deliver almost every gene modification by our Q-tech technology into almost any cell. Subsequently, it is also possible to use the platform for future applications, like the analysis of new non-coding RNAs.

IN VITRO: Can you give a profile of a representative customer?

CT: Our customers can be academic researchers, as well as scientists in research departments of biotech and pharmaceutical companies. The focus is clearly on cell-based assays and screenings.

IN VITRO: Munich is one of the major biotech centres in Europe. How important is this site for your company?

CT: Munich's bio-region is rich with biotechnology companies, universities, hospitals, the Max-Planck-Institutes and the Gene Center. It provided the ideal environment to establish our platform technology and continues to nurture its growth. The investments by the Bavarian state into this infrastructure allowed SIRION to set up virus production in a very short period of time.

IN VITRO: What are your plans for the near future?

CT: Our major aim is to market our Q-tech products throughout European and the world. This is our paramount goal for the upcoming years.

IN VITRO: What are the challenges you are currently addressing?

CT: We are planning to expand our production capabilities and, like any biotech company, we have to ensure our goals have a sound financial basis.

IN VITRO: Where do you see SIRION in 10 years?

CT: As I mentioned, we are aiming for the worldwide marketing of Q-tech as a standard tool for RNAi experiments. By virtue of our current and future innovations in viral transfection technology, we see SIRION becoming a valued partner to biotech and pharmaceutical companies in the development of vectors for therapeutic and industrial applications.

IN VITRO: SIRION is a young biotech company. What does Sigma-Aldrich mean to you?

CT: Because of our years in biochemical research, we were already quite familiar with Sigma-Aldrich. However, now that we are responsible for our business and the SIRION reputation, we realize that the quality of our products and services depends directly on the quality and reliability of our suppliers. We rely on Sigma-Aldrich to supply us with high-quality products so that we can do the same for SIRION customers. We also appreciate their understanding and attention to the needs of a small start-up business.

Molecular pathogenesis of hereditary inclusion body myopathy

Sabine Krause[1], Natalia Garcia-Angarita[1], Angelo Aleo[1], Maggie C. Walter[1], Philipp C. Janiesch[2], Johnny Kim[4], Thorsten Hoppe[4], Hanns Lochmüller[5] and Christian Thirion[1]

Introduction

Muscular dystrophy (MD) comprises a heterogeneous group of disorders that lead to a progressive loss of skeletal muscle mass. It is often associated with cardiac dysfunction. Patients with MD gradually become disabled and eventually may succumb to the disease. Currently there is no curative treatment for MD.

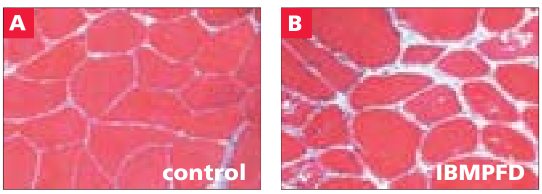
One form of MD is hereditary inclusion body myopathy (IBM). This form constitutes a group of adult-onset degenerative muscle disorders, inherited in recessive (IBM2/HIBM, OMIM 600737) or dominant traits (e.g. IBMPFD, OMIM 167320). IBM with Paget's disease of bone and Frontotemporal Dementia (IBMPFD) is caused by mutations in the valosin-containing protein gene (p97/VCP). The ubiquitin-selective chaperone *p97/VCP* is often required in protein degradation for substrate recruitment and ubiquitin chain assembly. In eukaryotes, p97/VCP is known to interact with UFD2a and CHIP homologues which regulate the myosin-directed chaperone UNC-45 involved in functional muscle formation.

Methods

Standard histology and H&E staining was performed as previously described (1). Primary human myoblasts were obtained from the Muscle Tissue Culture Collection (Munich, Germany) and were grown in skeletal muscle growth medium (PromoCell, Heidelberg, Germany) with 15% FCS. For immunofluorescence staining, myoblasts were adhered to coverslips coated with 10 µg/mL bovine collagen (Sigma Chemical Co., Taufkirchen, Germany). Myotubes were fused in differentiation medium (DMEM plus 2% heat-inactivated horse serum) on coverslips coated with 5 µg/mL laminin (Sigma). Cells were fixed in 3.7% paraformaldehyde in CSK buffer (10 mM PIPES, 100 mM NaCl, 300 mM sucrose, 16 mM EGTA, 26 mM $MgCl_2$ at pH 6.8) and permeabilised in 0.5% Triton X-100 in CSK buffer. Double immunofluorescence detection of human myoblasts and myotubes was described previously (2,3). Monoclonal antibodies and immunochemicals were purchased from Sigma and used as follows: anti-actinin (1:50, cat. no. A7811), anti-desmin (1:50, cat. no. D1033), anti-NCAM (1:50, cat. no. C9672), FITC-labelled phalloidin (1:100, cat. no. P5282). Goat anti-mouse Texas Red (DakoCytomation, Glostrup, Denmark) was used at 1:200. Anti-p97/VCP (1:25) was a generous gift from Thorsten Hoppe. Nuclei were visualised using bisBenzimide H 22358 (Fluka Chemical Co., Buchs, Switzerland).

Results

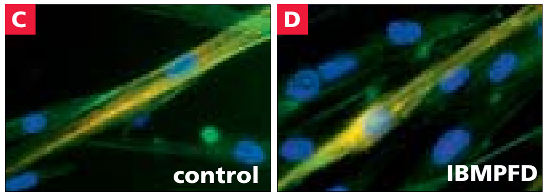
In IBMPFD patients, standard muscle histology of transverse muscle cryosections shows vacuolated fibres, variation in fiber size and increased proportion of connective tissue. Compared to normal skeletal muscle histology (figure A), a hallmark of IBMPFD is the presence of so-called rimmed vacuoles within muscle fibres (figure B).



Figures A and B Standard histology (H&E):
Figure A: Normal muscle.

Figure B: Muscle of IBMPFD patients. Note vacuolated fibers, variation of fibre size and increased connective tissue.

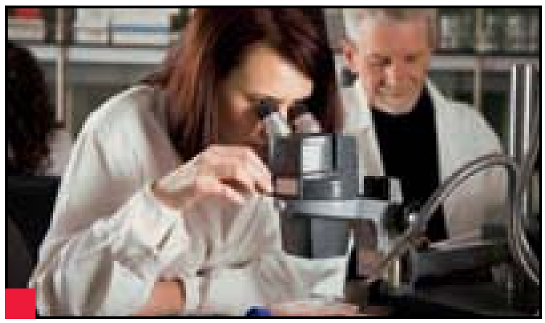
Our aim was to determine whether the regulation of p97/VCP is critical for human muscle development. Therefore, the subcellular distribution of p97/VCP was investigated by double immunostaining in primary myoblast cultures derived from human satellite cells. These are myogenic precursor cells that fuse to form terminally differentiated multinucleated myotubes during the process of myofibre differentiation and regeneration. As shown in figures C and D, p97/VCP is endogenously expressed in immature and fully differentiated myotubes.



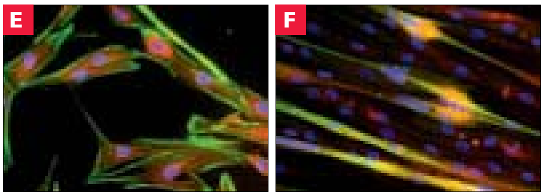
Figures C to H: Immunohistochemistry of myoblasts and in vitro differentiated myotubes as followed by conventional immunofluorescence microscopy.

Figure C: In human myoblasts, p97/VCP (red) is endogenously expressed in fully differentiated myotubes in the cytosol of wild-type and patient myoblasts, and is largely excluded from the nucleus. Positive desmin filament staining (green) relates to the myogenic lineage of the investigated cells.

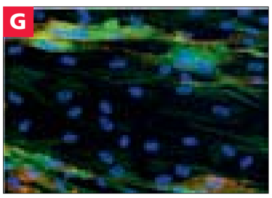
Figure D: Upon differentiation and myotube formation, p97/VCP (red) is expressed in human myotubes throughout the cytoplasm and in juxtanuclear distribution. Actin filaments are detected with FITC-phalloidin (green).



We examined whether myofibrillogenesis is altered in human p97/VCP mutant myotubes by immunofluorescence microscopy analysis using the myofibrillar marker phalloidin and the sarcomeric marker -actinin. As shown in figure E, myofibrils were formed and aligned correctly in wild-type cells (4). However, the myotubes of IBMPFD patients did not display actin banding or the typically ordered alignment of the-actinin positive myofibrillar array (figure F). The myogenic origin of the cells was verified by positive staining for the markers desmin and NCAM (figures G and H).



Figures E and F: Three days after fusion induction, double-fluorescence labelling of human differentiated, multinucleated myotubes with FITC-labelled phalloidin (green) and anti-sarcomeric actinin (red) revealed the absence of an ordered, compacted myofibrillar array in myotubes from a patient carrying a heterozygous R155C mutation in the *p97VCP* gene compared with control myotubes from an unaffected age-matched individual (wild type, WT) showing a characteristic sarcomeric array of labelled filaments.



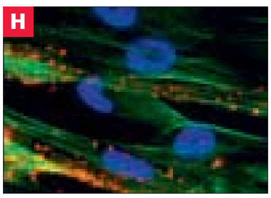


Figure G: Following myoblast fusion into multinuclear myotubes neural cell adhesion molecule (NCAM, red) is localised in patches on their surfaces.

Figure H: Detail from figure G showing NCAM immunoreactivity in small patches along differentiated myotubes 8 days after fusion induction. Actin filaments are labelled with FITC-labelled phalloidin (green). Nuclei were simultaneously visualised in blue (bisBenzimide H 33258, 40mg/ml).

Discussion

Muscle formation is a highly dynamic process governed by the turnover of regulatory and structural components. Remarkably, upon maturation and differentiation, myotubes of IBMPFD patients did not display actin banding or the typically ordered alignment of the -actinin positive myofibrillar array, suggesting a severe defect in myofibril organisation and sarcomere formation. In conclusion, we discovered an unanticipated function for p97/VCP in myofibre differentiation, maintenance and regeneration that is not present in terminally differentiated myotubes derived from satellite cells of muscle from IBMPFD patients. Future studies on the muscle-specific function of p97/VCP will help to understand the unexpected link between myofibre assembly and the molecular mechanism underlying inclusion-body formation in disease states like muscular dystrophy.

[1] Friedrich-Baur-Institute and Department of Neurology, Ludwig-Maximilians-University, 81377 Munich, Germany
[2] Centre for Molecular Neurobiology (ZMNH), University of Hamburg, 20251 Hamburg, Germany
[3] Institute of Human Genetics, Newcastle University, Newcastle upon Tyne, NE1 3BZ, Great Britain

References

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(4) Janiesch, P. C.; Kim, J.; Mouysset, J.; Barikbin, R.; Lochmuller, H.; Cassata, G.; Krause, S.; Hoppe, T. The ubiquitin-selective chaperone CDC-48/p97/VCP links myosin assembly to inclusion body myopathy. *Nat. Cell Biol.* 2007, 9, 379–390.

Staining Protocol Series: Sudan Black Staining System

Introduction

The reaction of neutrophil granules with the Sudan Black was first described by Sheehan and Storey in 1947.[1] Several lipids including phospholipids, neutral fats and sterols are stained intensely by Sudan Black B. The leukocyte Sudan Black B staining pattern usually parallels that of myeloperoxidase.[2] Cells committed along lymphoid pathways display negative stain, whereas myeloid and monocytoid forms display characteristic positive reactions. Thus Sudan Black B is considered a useful adjunct in the identification of myelocytic and myelomonocytic leukemias.[2]

Previous methods utilised formaldehyde vapour fixation of blood films.[2] This technique may result in cell loss and staining artefacts. The Sigma-Aldrich procedure utilises a buffered glutaraldehyde fixative and shortened incubation time that results in excellent staining without cellular loss or distortion.[3]

Sigma-Aldrich Sudan Black B Staining System is for use in Histochemical Demonstration of Neutrophil Granules in Blood or Bone Marrow Films. The Sudan Black B Staining System is for "In Vitro Diagnostic Use".

Reagents

SUDAN BLACK B STAINING REAGENT
(Cat No. 380-1) Sudan Black B, 0.18% (w/v), in 69% ethanol and containing phosphate-buffered phenol.

HEMATOXYLIN SOLUTION, GILL NO. 3
(Cat. No. GHS-3) Certified hematoxylin, 6 g/l, sodium iodate, 0.6 g/l, aluminum sulphate, 52.8 g/l and stabiliser.

GLUTARALDEHYDE SOLUTION
(Cat. No. 380-2) Glutaraldehyde, 4% in borate buffer, pH 7.6.

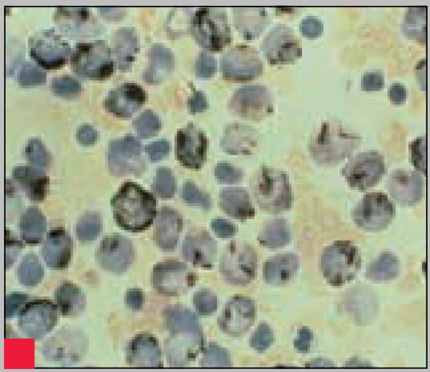
Reagent Preparation

Glutaraldehyde fixative is prepared by adding 25 mL reagent grade acetone to 75 mL Glutaraldehyde solution. Tightly cap and store at 2-8 °C. Stable for several months but discard if turbidity develops.

Procedure

1. Fix smears or films in chilled Glutaraldehyde fixative (2-8 °C) for 1 minute with gentle agitation.
2. Rinse thoroughly in deionised water.
3. Stain slides in Sudan Black B reagent for 5 minutes using intermittent agitation.
4. Rinse slides 3 or more times in 70% ethanol until no more dye washes out.
5. Rinse slides thoroughly in deionised water.
6. Counterstain in Gill's Hematoxylin No. 3 for 5 minutes.
7. Rinse slides thoroughly in tap water and allow to air dry.
8. Coverslip slides with permanent mounting media.

Results



Neutrophils and their precursors show blue-black intracellular granulation. Monocytes stain less intensely and lymphocytes do not stain with Sudan Black B.

References

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Staining Protocol Series: Naphthol AS-D Chloroacetate Esterase 90 (Capsule)

Introduction

Cellular esterases are ubiquitous and appear to represent a series of different enzymes acting upon select substrates. Under defined reaction conditions, it may be possible to determine hemopoietic cell types, using specific esterase substrates. The described methods provide means to distinguish granulocytes from monocytes.

To perform the test, blood, bone marrow films or tissue touch preparations are incubated with naphthol AS-D chloroacetate in the presence of a stable diazonium salt. Enzymatic hydrolysis of ester linkages liberates free naphthol compounds. These couple with the diazonium salt, forming highly coloured deposits at the sites of enzyme activity.

Sigma-Aldrich reagents are intended for the cytologic demonstration of naphthol AS-D chloroacetate esterase and -naphthyl acetate esterase in blood, bone marrow films or tissue touch preparations. Esterase reagents are for "In-Vitro Diagnostic Use".

Reagents

SUDAN BLACK B STAINING REAGENT
(Cat No. 380-1) Sudan Black B, 0.18% (w/v), in 69% ethanol and containing phosphate-buffered phenol.

HEMATOXYLIN SOLUTION, GILL NO. 3
(Cat. No. GHS-3) Certified hematoxylin, 6 g/l, sodium iodate, 0.6 g/l, aluminum sulphate, 52.8 g/l and stabiliser.

GLUTARALDEHYDE SOLUTION
(Cat. No. 380-2) Glutaraldehyde, 4% in borate buffer, pH 7.6.

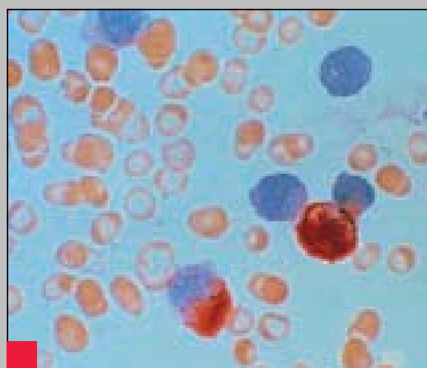
Reagent Preparation

Naphthol AS-D Chloroacetate solution is prepared by dissolving 1 capsule in 2 mL Dimethyl Formamide. (Prepare immediately prior to use.) TRIZMAL 6.3 Buffer solution is prepared by diluting 1 part concentrate with 9 parts deionised water. Citrate Dilute solution is prepared by diluting 1 part concentrate to 9 parts deionised water. Citrate-Acetone-Methanol Fixative: take 18 mL Citrate Dilute solution and add 27 mL Acetone, and 5 mL Methanol. Seal tightly and discard after 8 hours.

Procedure

1. Pre-warm diluted TRIZMAL 6.3 Buffer solution to 37 °C.
2. Fix slides for 1 minute in Citrate-Acetone-Methanol fixative at room temperature.
3. Wash thoroughly in deionised water and air dry for 20 minutes.
4. Add 1 capsule of Fast Corinth V Salt to 50 mL TRIZMAL 6.3 Dilute Buffer while stirring constantly.
5. When salt is completely dissolved in buffer, add 2 mL Naphthol AS-D Chloroacetate. Continue mixing for 15–30 seconds. Do not filter.
6. Add slides and incubate for 5 minutes at 37 °C. Protect from light.
7. Remove slides and wash in deionised water for 3 minutes.

Results



This enzyme is considered specific for cells of granulocytic origin with cells showing red granulation. Activity is weak or absent in monocytes and lymphocytes.

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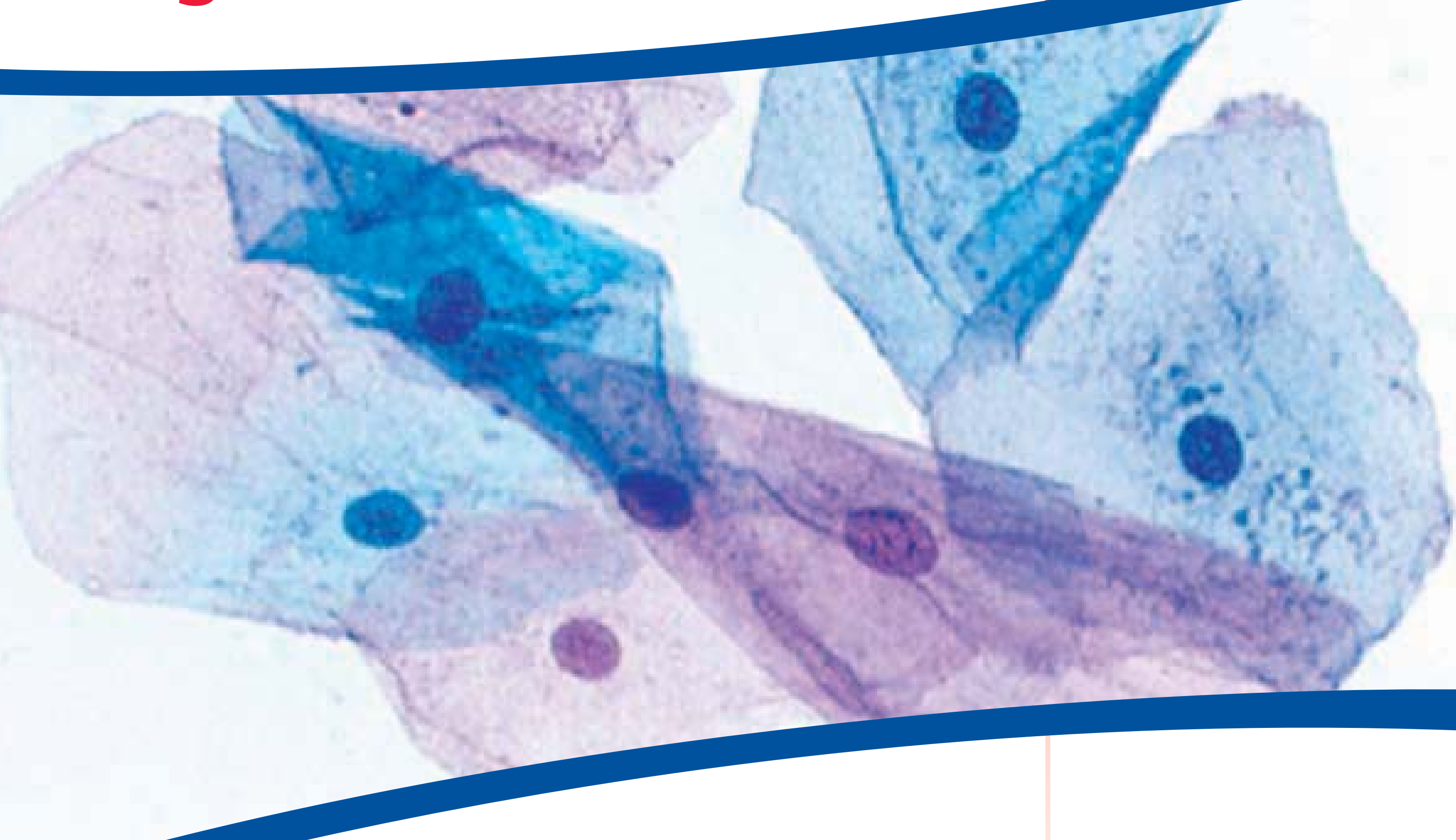

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HT40332-1L	for *in vitro* diagnostic use	
HT40380-2.5L	for *in vitro* diagnostic use	

Hematoxylin Solution, Gill No. 2		
GHS232-1L	for *in vitro* diagnostic use	Discount 45%
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Combined UCHL-1/PASH Staining in the Diagnosis of Renal Transplant Rejection

Ranjitha Veerappan, MD, Lou Ellen Miller, HT (ASCP), Piyush Joshi, MD and George A. Youngberg, MD

Determining if the cause of renal allograft dysfunction in the early post transplant period is due to rejection or other reasons is a daunting task for the clinician. Clinical signs and symptoms alone are not helpful in clearly distinguishing the numerous causes of renal dysfunction. Various non-invasive tests including imaging studies have also been unsuccessful in distinguishing rejection from other causes.[1] Renal allograft biopsy has, therefore, emerged as a key element in aiding in the accurate diagnosis of transplant rejection.

An allograft biopsy in the context of acute rejection not only helps to confirm the diagnosis, but it also reveals if the rejection is a result of humoral (antibody-mediated) or cellular immunologic mechanisms. This distinction determines the therapeutic course and the successful reversal of acute rejection. Cellular rejection is the more common type and is typically manifested by a T-lymphocyte attack directed against renal tubules (and occasionally against blood vessels). Microscopically, the early stages are characterised by oedema and focal infiltration of the interstitium and peritubular capillaries by lymphocytes. In later stages, plasma cells, monocytes, and macrophages appear along with a more diffuse lymphocytic infiltrate. Invasion of the tubular epithelium by lymphocytes is a characteristic finding of tubulointerstitial rejection.[2] To standardise diagnoses and avoid false positives, a formal system for interpreting renal allograft biopsies was developed.[3] In this system, the diagnosis of acute cellular tubulointerstitial rejection is based on the finding of tubulitis. The criterion for diagnosing tubulitis is the identification of intraepithelial lymphocytes in the tubules.

The interpretation of tubulitis has traditionally been done using periodic acid-Schiff hematoxylin (PASH) staining, which clearly identifies the tubular basement membranes and, therefore, aids in the accurate assessment of the location of the lymphocytes (inside versus adjacent to the tubule). However, a significant drawback related to the PASH stain is that intraepithelial lymphocytes cannot always be reliably distinguished from tubular epithelial cell nuclei. Additional stains such as leukocyte common antigen (LCA) or a T-cell stain (UCHL-1) that specifically identifies lymphocytes have been used to address this issue. However, these stains again raise the issue of the location of the lymphocytes (inside versus adjacent to the tubule), since the basement membrane cannot be visualised by these methods. The ideal stain, therefore, would be the one that combines the ability of the PASH stain to visualise the basement membrane and also identify the lymphocytes with a specific marker such as LCA or UCHL-1. This method would greatly facilitate and improve the accuracy of diagnosing acute cellular tubulointerstitial rejection. Such a stain has recently been developed[4] and can be produced using the Ventana Benchmark automated immunohistochemistry staining system and the NexES Special stains instrument.

Case Report

A 32-year-old man with a live unrelated donor renal allograft was noted to have rising creatinine. His immunosuppressive regimen consisted of rapamycin and steroids, with no mycophenolate mofetil or calcineurin inhibitors. The elevated creatinine did not respond to pulse methylprednisolone, so a percutaneous renal biopsy was obtained.

Methods

Routine light microscopic renal biopsy stains, including PASH and Hematoxylin & Eosin (H&E), were made using standard methods. A UCHL-1/PASH stain was prepared according to the method of Resch et. Al,4 using a Ventana (Ventana, Tucson, AZ) combined automated immunohistochemical (Benchmark™) and histochemical (NexES) stainer. Briefly, the renal core biopsy was received in Carson's fixative, processed overnight, and paraffin embedded. The sections were cut at 3 microns and stained on the Benchmark using the iView DAB paraffin detection kit (Ventana, Tucson, AZ) with the T-cell (CD45RO) antibody. After the antibody step, the slides were removed from the Benchmark, rinsed in DI water/Dawn dishwashing liquid, and placed on the NexES for routine PASH stain. The slides were then dehydrated through graded alcohols, cleared in xylene, and mounted with a permanent mounting medium.

Results

The biopsy demonstrated moderate interstitial inflammation involving more than 25% of the cortical area. The inflammatory infiltrate consisted predominantly of lymphocytes, with some admixture of plasma cells, eosinophils, and neutrophils. Several tubular profiles showed more than four mononuclear cells per tubular cross section. This was most clearly demonstrated on the PASH/T-cell stain (figure 1). No tubular cross sections contained more than ten mononuclear cells. The PASH/T-cell stain confirmed that the majority of lymphocytes present were T-cells. A diagnosis of acute cellular rejection, tubulointerstitial type was made (Banff 1997 acute rejection, type IA).

1

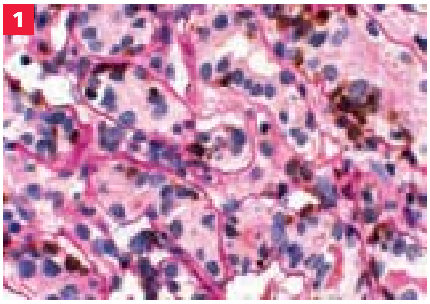

Figure 1: Tubulitis in the renal biopsy. Lymphocyte cytoplasm stains brown. Tubular borders are clearly delineated. UCHL-1/PASH 400x

Discussion

The key element in making a diagnosis of acute cellular rejection, tubulointerstitial type, is the identification of tubulitis. This can be challenging because lymphocytes in foci of presumptive tubulitis can be difficult to distinguish from tubular nuclei on standard stains like H&E or PASH (figure 2).

2

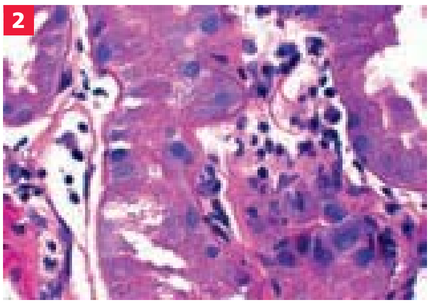

Figure 2: Distinguishing lymphocytes from tubular cells can be difficult on a standard PASH stain. PASH 400x

Immunohistochemical stains for LCA or T-cell markers can reliably identify the lymphocytes, but it may be difficult for these stains to distinguish whether the lymphocytes are truly inside the tubular basement membrane or merely adjacent to the tubule (figure 3).

3

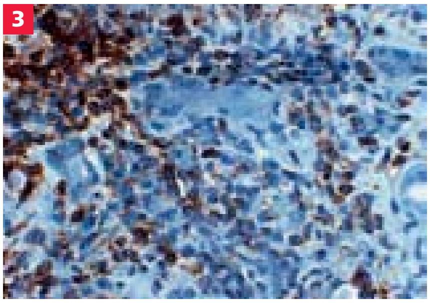

Figure 3: It is difficult to identify borders of tubules on a standard lymphocyte stain; determining the location of the lymphocytes (outside versus inside the tubular basement membranes) can be problematic. LCA 400x

Combining a basement membrane stain (PAS) with a T-cell stain (UCHL-1) elegantly solves the problem of both specifically identifying T-lymphocytes and precisely determining their location (figure 4). Thus, the presence of tubulitis can quickly and accurately be confirmed or excluded. This combination of UCHL-1/PASH greatly improves the accuracy of diagnosis of acute cellular tubulointerstitial rejection and helps determine the therapeutic course and prognosis.

4

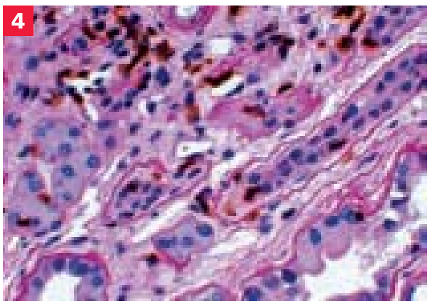

Figure 4: Lymphocytes are clearly identified, and their precise location in relation to tubular basement membranes is easy to determine on the PASH/T-cell stain. UCHL-1/PASH 400x

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Atto labels and their conjugates – versatile, bright and stable tools for imaging

Atto dyes are a comprehensive series of fluorescent dyes, covering the entire spectrum of visible light and matching the most common output wavelengths of excitation light sources, especially mercury and xenon lamps, but also common lasers (**table 2**). Atto dyes provide brightest fluorescence with narrow fluorescence emission spectra. These properties enable the parallel imaging of different targets in cells, tissues or other biological samples. **Figure 2** shows characteristic absorbance and emission spectra of the atto dye family.

Atto 488 is a superior alternative to widely used fluorescein, providing a tremendously improved photostability and brighter fluorescence. It can be excited with the same light sources as fluorescein dyes or Alexa 488, and the same optical filter sets and instrument settings are used to record the emission. Proteins can be labelled with numerous Atto 488 labels without significant quenching. For longer wavelengths, Atto 550 and Atto 565 are efficiently excited by HeNe lasers (543 nm) and are used as alternatives to rhodamine dye, Cy™ 3 or Alexa 550, offering high brightness and very good photostability. Atto 635, 647 or Atto 655 are well suited for excitation with HeNe or Krypton lasers, similar to Cy™ 5 or Alexa 555.

Autofluorescence of biological samples or support media can be a serious limitation for sensitivity and specificity of fluorescent techniques like immunohisto-chemistry and ELISA assays. As most of the fluorescence of biological samples comes from shorter wavelengths, the impact of autofluorescence decreases with longer excitation and detection wavelengths. Similar holds for most of the autofluorescence of solvents, glass or polymer supports. With excitation maxima up to 740 nm and emission maxima up to 764 nm, Atto dyes provide a set of tools to circumvent problems with autofluorescence.

Atto labels are available as reactive succinimidyl esters and maleimides, enabling straightforward coupling by common procedures. For additional information on individual products please visit our homepage under **www.sigma-aldrich.com**

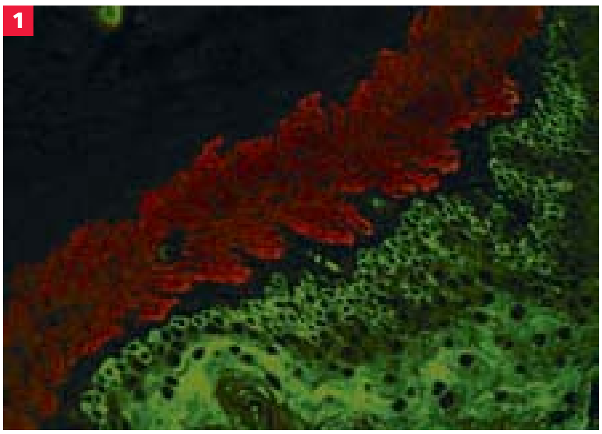
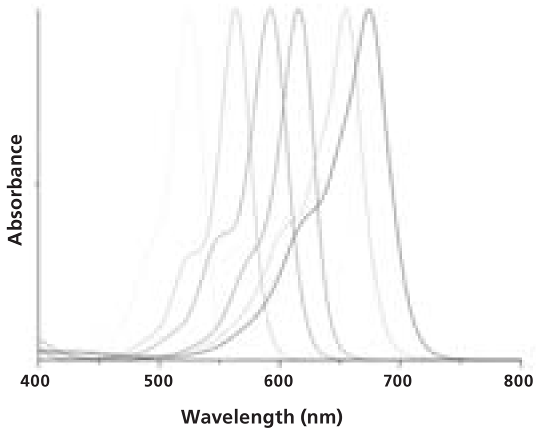


Figure 1: Rat stomach: Actin stained with mouse anti-smooth muscle-actin antibody and Atto 488 anti-mouse IgG (green), cytokeratin stained with polyclonal rabbit anti-cytokeratin and Mega485 anti-rabbit IgG (yellow). Both labels are excited by just one laser (Argon laser).



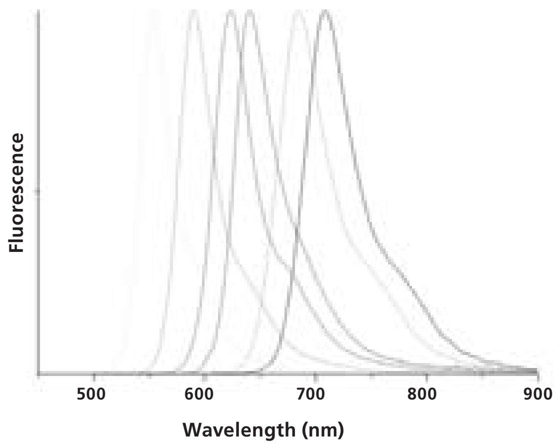


Figure 2: Absorption and emission spectra of Atto 520, Atto 565, Atto 590, Atto 610, Atto 655 and Atto 680

Label	Goat anti-mouse Ig	Goat anti-rabbit IgG	Goat anti-human IgG	Goat anti-chicken IgG
Atto 488	18772	62197	52526	
Atto 550	43394	44328		
Atto 590		68919		50913
Atto 647	50185	40839		
Mega 485	12708	38376		
Mega 520	39304	02295		

Table 1: Antibody conjugates labelled with new Atto or Mega labels

Name	λ_{max} abs [nm]	λ_{max} em [nm]	ηfl [%]	λ_{max} [l/mol cm]	Suitable laser excitation sources	Spectral match to
Atto 465	449	503				
Atto 488	501	523	80	90000	Ar laser	FITC, Alexa 488
Atto 495	499	535	45	80000	Ar laser	
Atto 520	525	545	90	110000	Ar laser	JOE
Atto 532	532	553	90	115000		Alexa 532
Atto 550	554	576	80	120000	HeNe laser	TRITC, Cy3™, Alexa 546
Atto 565	563	592	92	120000	HeNe laser	Rhodamine B, Lissamine, Alexa 555
Atto 590	598	634	80	120000	HeNe laser	Texas Red, Alexa 594
Atto 594	601	627	85	120000	HeNe laser	Texas Red, Alexa 594
Atto 610	605	646	70	15000		Alexa 610
Atto 620	620	641	50	120000		
Atto 633	629	657	64	130000	HeNe laser, Ruby laser	Alexa 635
Atto 635	635	659	25	120000	HeNe laser, Ruby laser	Alexa 635
Atto 647	645	673	20	120000	HeNe laser, Kr laser, Ruby laser,	Cy5™, Alexa 647
Atto 655	665	690	30	125000	HeNe laser, Kr laser	
Atto 680	680	702	30	125000	HeNe laser, Kr laser, Ruby laser, GaInP laser	Cy5.5™, Alexa 680
Atto 700	740	764	10	120000	Ruby laser	Alexa 700

Table 2: Atto labels and their spectroscopic properties

Antibody and other fluorescent conjugates:

We optimised the labelling of antibodies based on the innovative series of atto labels, to provide high-quality conjugates with ideal brightness and low background.

Secondary antibodies, the general workhorses for immunochemistry, are offered as conjugates with several of our atto labels as well as Mega labels, which are characterised by a large gap between excitation and emission maxima (**tables 1 and 3**). For most of the atto labels, streptavidin and Biotin conjugates are also available.

Multiple staining with single excitation light:

Parallel staining of different structures or target molecules in biological tissues or other samples can be complicated by overlaps of fluorescence signals. Thus labels with clearly distinct emission spectra are preferred. For this purpose, the wide spectrum of labels offers a variety of suitable combinations (**figures 1–4**).

Where laser light is used for excitation, as in the case of confocal microscopy, the suitable excitation of labels with distinct emission spectra may require two or more different lasers. This is because for most common labels (e.g. FITC, TRITC, Cy3™, Cy5™, Alexa labels) excitation and emission maxima are relatively close. As the laser wavelengths are far away from the maximum excitation of these dyes, fluorescence imaging works at a low efficiency. Each additional laser source increases costs significantly, thus multiple fluorescent staining with just one excitations source would be a convenient and cost-efficient methodology and provide increased flexibility.

For additional information on individual products, please visit our homepage at www.sigma-aldrich.com

Another potential problem with multiple fluorescent staining is the overlap of emission wavelength of dye (A) with excitation of dye (B), resulting in a lower signal intensity of dye A.

In contrast to the above-mentioned labels, Mega labels are characterised by a large gap between excitation and emission wavelength (**table 3**). All of them can be excited with argon lasers or other widely used light sources, but their fluorescence varies. Mega labels, if combined with atto dyes, enable the visualisation of different structures with just one excitation source. **Figure 1** shows an application of an Atto 488 and a Mega 485-labelled antibody in confocal microscopy. But such combinations can also be used on conventional fluorescence microscopes, using mercury lamps or even standard filter sets optimised for conventional fluorophores **(figure 3)**.

Name	λ_{max} abs [nm]	λ_{max} em [nm]	λ_{max} [l/mol cm]
Fluorescent Red Mega 480	480	640	40000
Fluorescent Red Mega 485	485	559	20000
Fluorescent Red Mega 500	500	612	90000
Fluorescent Red Mega 520	520	664	50000

Table 3: Mega labels and their spectroscopic properties

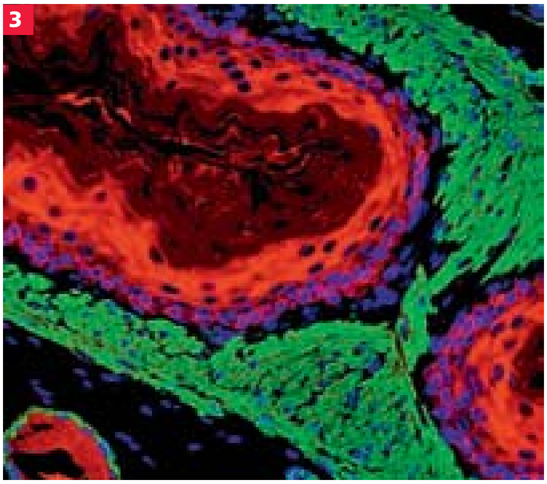


Figure 3: **Rat stomach: Actin stained with mouse anti-smooth muscle-actin antibody and Atto 488 anti-mouse IgG (green), cytokeratin stained with polyclonal rabbit anti-cytokeratin and Mega520 anti-rabbit IgG (red), counterstained with DAPI (blue). Courtesy of Jacob Zbaeren, Inselspital Bern, Switzerland**

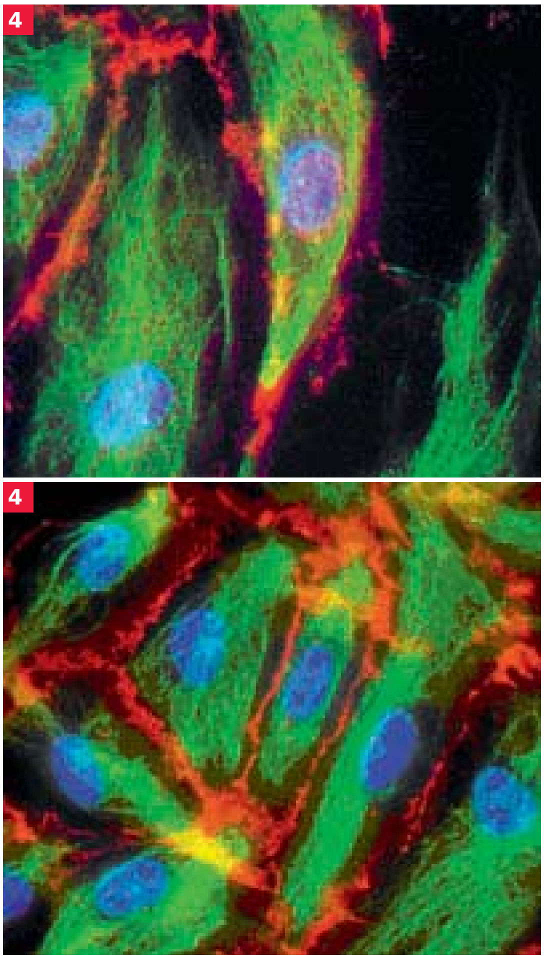


Figure 4: **Human endothelial cells: Vimentin stained with mouse anti-vimentin and Atto 550 anti-mouse IgG (green), cadherine stained with rabbit anti-cadherine antibody and Atto 655 goat anti-rabbit IgG (red). Counterstained with DAPI (blue).**

Stability

For well-known and commonly used labels like fluoresceins, photostability is limited. In various applications, esp. in immunofluorescence, bleaching fluorescence intensity is a major concern, limiting quality and sensitivity of imaging. Photostability becomes even more important with the increasing use of laser excitation, confocal and two-photon illumination, and the increasing sensitivity of methods down to the single molecule level. Also the tracking of processes over time in living cells requires stable dyes.

Atto labels, in contrast to some of the most widely used dyes, have more rigid structures, which makes them more photostable. Atto conjugates are exceptionally stable, in several cases even outperforming the dyes, that were considered the best choice in place until now.

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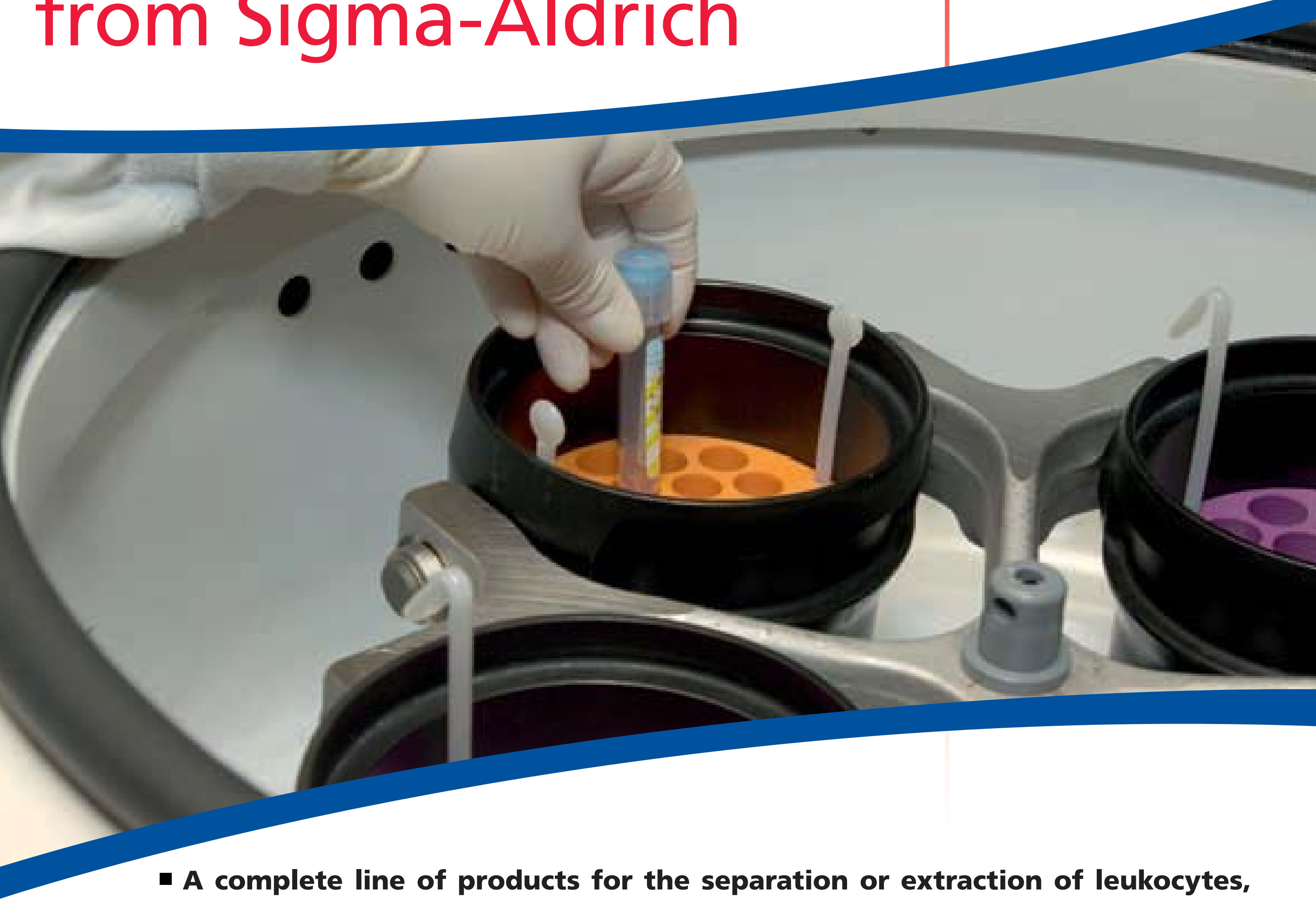

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Pseudomonas Media and Tests

Detection, identification, differentiation and cultivation of *Pseudomonas* species

Figure 1: HiFluoro *Pseudomonas* Agar under UV light

Pseudomonas are motile (one or more polar flagella), rod shaped and aerobe Gram-negative bacteria. They are found almost everywhere, in soil, water, plants and animals. In most cases it is not pathogenic and in fact can be beneficial. For example, *P. putida* is used as a bio-scrubber to aid in the biodegradation of diverse organic compounds in polluted air and waste water. However, *P. aeruginosa* is an infamous opportunistic human pathogen most commonly affecting immunocompromised patients. Along with *P. maltophilia*, it accounts for the majority of human infections. Pathogenic *Pseudomonas* are found throughout the body, most commonly in the urinary tract, respiratory tract, blood and wounds (1).

Rugged and opportunistic, *Pseudomonas* use a wide range of nutritional sources, even very simple nutritional environments without any organic compounds. They can remain viable for long periods of time in many different habitats and under very adverse conditions. They are also widespread, being found in water, saline solutions, utensils and even in cosmetics, pharmaceuticals and disinfectants, and many natural and manufactured foods. Psychrotrophic (cold-tolerant) *Pseudomonas* species are a significant food spoilage problem in refrigerated meat, fish, shellfish and dairy products. Because *Pseudomonas* thrive in water systems, they can be a source of contamination in the food and beverage industry (2).

Pseudomonas are not generally fastidious microorganisms. They can grow on very simple media like Kind Agar, for example, which contains a protein hydrolysate, magnesium chloride, potassium sulphate and agar. Analytical microbiology leverages a microbe's unique biochemistry to aid in its identification. For example, selective *Pseudomonas* media use cetrimide, nalidixic acid, cephaloridine, penicillin G, pimaricin, malachite green and other inhibitory agents. The proteolytic activity, lipolytic activity, fluorescent pigment formation, nitrate utilisation, glutamate utilisation, hemolytic reaction and other biochemical reactions are used in the media for the identification and differentiation of Pseudomonas species.

Pseudomonas gives negative Voges Proskauer, indole and methyl red tests, but a positive catalase test. While some species show a negative reaction in the oxidase test, most species, including *P. fluorescens*, give a positive result (**figure 2**). Another feature associated with *Pseudomonas* is the secretion of pyoverdin (fluorescein, a siderophore), a fluorescent yellow-green pigment under iron-limiting conditions (3). Certain *Pseudomonas* species may also produce additional pigments, such as pyocyanin (blue pigment, a siderophore) by *P. aeruginosa* (4), quinolobactin (yellow, dark green in presence of iron, a siderophore) by *P. fluorescens* (5), a reddish pigment called pyorubrin and pyomelanin (brown pigment). On blood agar a hemolytic reaction can be observed.

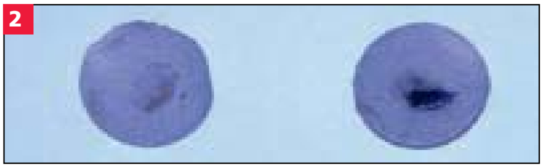

Figure 2: Oxidase test

Negative control

Scientific classification of *Pseudomonas*:

Kingdom: Bacteria	Order: Pseudomonadales
Phylum: Proteobacteria	Family: Pseudomonadaceae
Class: Gamma Proteobacteria	Genus: Pseudomonas

Pseudomonas utilises sugars as an energy source by using the Entner-Doudoroff pathway with pyruvate as the end product (dissimilation). The reaction utilises a different set of enzymes from those used in glycol ysis and the pentose phosphate pathway. Fermentation catabolism is not observed in *Pseudomonas*, but some species, like *P. aeruginosa, P. stutzeri and P. denitrificans*, are able to use nitrate as an electron acceptor instead of oxygen. Growth can also occur under anaerobic conditions when the denitrification pathway is used.

Sigma-Aldrich supplies a wide array of products for the detection, identification, differentiation, enumeration and cultivation of *Pseudomonas*, using its biochemical characteristics, including Gram staining kit, and many types of selective growth media (**table 1**) and diagnostic tests (**table 2**). Additional information on media and tests for *Pseudomonas* and a wide range of other microbes can be found on our website:
www.sigma-aldrich.com/microbiology

Jvo Siegrist, Product Manager Microbiology ivo.siegrist@sial.com

Cat. No.	Brand	Nonselective Broths
A0465	Sigma	Alternative Thioglycollate Medium
53286	Fluka	Brain Heart Broth
B5051	Sigma	Bushnell H aas Broth
D3435	Sigma	Dey-Engley Neutralizing Broth
63649	Fluka	Membrane filter Rinse Fluid (USP)
70149	Fluka	Nutrient Broth No. 3
03856	Fluka	Nutrient Broth No. 4
70179	Fluka	Peptone Water
77187	Fluka	Peptone Water, phosphate-buffered
40893	Fluka	Peptone Water, phosphate-buffered, Vegitone
70157	Fluka	Thioglycollate Broth (USP Alternative)
41960	Fluka	Vegitone Infusion Broth
Cat. No.	**Brand**	**Selective Enrichment Broths & Biochemical Identification Broths**
00185	Fluka	Acetamide Nutrient Broth
17129	Fluka	Asparagine Proline Broth
78886	Fluka	Cetrimide Broth
63163	Fluka	Malachite Green Broth
39484	Fluka	Methyl Red Voges Proskauer Broth
14305	Fluka	Motility Nitrate Medium
72548	Fluka	Nitrate Broth

Cat. No.	Brand	Nonselective Agars for Cultivation, Enumeration and Isolation
70147	Fluka	Milk Agar
70148	Fluka	Nutrient Agar
44776	Fluka	Nutrient Agar Plates (Diameter 55 mm)
80957	Fluka	Plate Count Skim Milk Agar
17209	Fluka	R-2A Agar
17175	Fluka	Skim Milk Agar, modified
51414	Fluka	Tryptic Soya Agar with Polysorbate 80 and Lecithin
70159	Fluka	Tryptone Glucose Extract Agar
T2188	Sigma	Tryptone Glucose Yeast Extract Agar
01497	Fluka	Yeast Extract Agar
Cat. No.	**Brand**	**Nonselective Agars for Differentiation**
70133	Fluka	Blood Agar (Base)
21065	Fluka	Calcium caseinate Agar
55420	Fluka	CLED Agar
16636	Fluka	HiCrome (™) UTI Agar, modified
60788	Fluka	King Agar A
60786	Fluka	King Agar B
75315	Fluka	OF Test Nutrient Agar
P1852	Sigma	*Pseudomonas* Agar (for Fluorescein)
91015	Fluka	Tributyrin Agar

Cat. No.	Brand	Selective Agars for Detection and Isolation
11012	Fluka	Cetrimide Nalidixic acid Agar
22470	Fluka	Cetrimide Agar
70887	Fluka	Cetrimide Agar
P2102	Sigma	*Pseudomonas* Agar Base
14521	Fluka	Cetrimide Agar Plates (Diameter 55 mm)
17168	Fluka	Milk Agar, modified according to Brown & Scott
17208	Fluka	*Pseudomonas* Isolation Agar
Cat. No.	**Brand**	**Selective Agars with Differential System for Differentiation, Detection and Isolation**
50875	Fluka	GSP Agar
78996	Fluka	HiFluoro (™) *Pseudomonas* Agar Base

Table 1: Media for *Pseudomonas*

Cat. No.	Brand	Diagnostic Tests for Pseudomonas
88597	Fluka	Catalase Test
05686	Fluka	DMACA Indole Disks
49825	Fluka	DMACA Reagent
78719	Fluka	Kovac's Reagent Strips
60983	Fluka	Kovac's Reagent for indoles
67309	Fluka	Kovac's Reagent for indoles
08714	Fluka	Methyl Red Solution
70439	Fluka	Oxidase Test
40560	Fluka	Oxidase Strips
07345	Fluka	Oxidase Reagent acc. Gaby-Hadley A
07817	Fluka	Oxidase Reagent acc. Gaby-Hadley B
18502	Fluka	Oxidase Reagent acc. Gordon-McLeod

Table 2: Test for identification and differentiation of Pseudomonas **w**

References:

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Assessment of the fatty acid status in blood lipids

Kits provide means for easy sample collection and derivatization

Valuable diagnostic information can be obtained from the fatty acid profile of a single drop of blood.

1

Fatty acids perform diverse biological functions. They are important as dietary energy sources, and as components of plasma, cell lipids and cell membranes. In the diet, their content varies both in amount (quantitatively) and in composition or profile (qualitatively).

Classifications of fatty acids based on degree of unsaturation

Fatty acids (FA) are carbon chains, generally with an even number of carbon atoms and a varying number of double bonds. FA with zero double bonds are the saturated FA (SFA). These are found primarily in animals, especially ruminants and derived products, and in some vegetable fats, like coconut and palm oils. FA with one double bond are the monounsaturated FA (MUFA).

The most important MUFA is oleic acid (C18:1), which is present in both vegetable oils (olive and canola) and animal fat (pork and poultry). FA with two or more double bonds are the polyunsaturated FA (PUFA). The 18C components of the PUFA class are the so-called essential FA (EFA) that cannot be synthesised by animals. EFA are synthesised by plants and are therefore found in vegetable fats. PUFA with longer chains (>20 carbon atoms) and a higher degree of unsaturation (>3 double bonds) are almost exclusively animal derived.

The PUFA are further subdivided in two major series: the omega-6 (or ω-6) and the omega-3 (or ω-3), depending upon the number of carbon atoms between the methyl end of the FA chain and the nearest double bond. The long-chain, highly unsaturated omega-6, especially arachidonic acid (AA, C20:4), and omega-3, especially eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6), play important structural and functional roles in cell membranes. While some AA is found in lean animal tissues, especially meat, EPA and DHA are mainly found in fatty fish where they accumulate following the consumption of algae.

Fatty acids and health

There is a vast amount of literature suggesting that high intake of omega-3 FA is associated with higher health status. However, the optimal omega-3 levels are generally higher than provided by the typical Western diet. The correlation with high omega-3 intake and health is thought to be due to prevention of the onset of chronic degenerative, inflammatory processes of the cardiovascular and respiratory system, among other benefits. Because of the health benefits of the presence of "good" FA, and the deleterious effects of high levels of "bad" FA, the FA status in blood is commonly measured.

The following three relevant points are notable:

- FA, especially PUFA, are mainly derived from the diet.
- FA intake is difficult to assess on the basis of food composition data and questionnaires, especially the omega-3 FA because of their low levels in the diet (<0.5 g omega-3 vs. >100 g total FA/day).
- The FA status is an indicator of FA intake and also correlates with various patho-physiological conditions.

Recommended dietary strategies for improving the health status and prevention and treatment of diseases are typically aimed at increasing omega-3 FA levels, especially the EPA and DHA.

Uncomplicated collection of blood samples for assessment of fatty acid status

Monitoring a patient's FA profile is important to optimise fat intake and verify the adherence to and the efficacy of dietary strategies aimed at improving health. Sufficient data to make such assessments can be obtained from only a small drop of fingertip blood (figure 1).

Sigma-Aldrich has created a convenient kit for the easy collection of blood drops, their storage, shipment and processing for FA analysis. A separate derivatisation kit is available for the GC analysis of the sample. Information obtained through subsequent analysis of the FA in the blood samples allows the definition of the FA status of an individual in relation to the average values in the population and provides the basis for the development and application of adequate preventative dietary strategies.

Cat. No.	Brand	Description	Pack Size
11312	Fluka	Blood Collection Kit Includes blood collection dipsticks, desiccant packs, foil-barrier ziplock bags, 50 mL BHT solution and complete instructions. Enough for 100 tests.	1 kit
05904	Fluka	Derivatization Kit Includes methanolic HCl solution (1.25 M), saturated KCl solution, distilled water and working instruction sheet. Enough for 100 tests.	1 kit

Table 1: Valuable diagnostic information can be obtained from the fatty acid profile of a single drop of blood.

Reference

Marangoni F., Colombo C., Galli C. *Anal. Biochem.* 2004, 326, 267–72.

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