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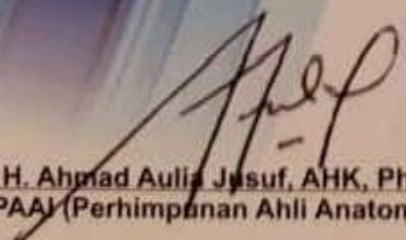
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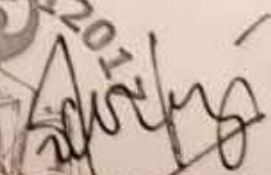
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has participated as **SPEAKER**  
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THEME "FROM BASIC TO CLINICAL APPLICATION"**

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Chairman of the Committee

Speaker 8 SKP, Participant 6 SKP, Moderator 2 SKP, Committee 2 SKP  
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# ANNUAL SCIENTIFIC MEETING

PERHIMPUNAN AHLI ANATOMI INDONESIA (PAAI)  
"FROM BASIC TO CLINICAL APPLICATION"

MAKASSAR, 9 - 12 AGUSTUS 2017

Editor:

dr. M. Iqbal Basri, Sp.S

dr. Rahmawati Mihnajati, Ph.D, Sp.PD

dr. Asty Amalia



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**DAFTAR ISI**  
**PERTEMUAN ILMIAH NASIONAL**  
**PERHIMPUNAN AHLI ANATOMI INDONESIA**  
**2017**

Sampul	i
Kata Pengantar Dekan Fakultas Kedokteran Universitas Hasanuddin	iii
Sambutan Ketua Pengurus Besar Perhimpunan Ahli Anatomi Indonesia	iv
Kata Pengantar Ketua Panitia Pertemuan Ilmiah Nasional Perhimpunan Ahli Anatomi Indonesia	vi
Susunan Acara Pertemuan Ilmiah Nasional Perhimpunan Ahli Anatomi Indonesia	vii
Daftar Isi	viii
<b>Kumpulan Materi Plenary Session</b>	
Research Trends in Histology..... <i>Ahmad Aulia Jusuf</i>	2
Challenges And Future Directions Of Anatomy: Advanced Study Of Brain Microcircuitry Connections ..... <i>Taruna Ikrar</i>	4
Inovasi Pembelajaran Anatomi..... <i>Sigit Moerjono</i>	7
Basic Anatomy: Teaching Methodology and Innovation..... <i>Fundhy Sinar Ikrar Prihatanto</i>	9
The Drivers for Evolution..... <i>Eiichi Hondo</i>	12
The Role of Skull Anatomic Landmarks in Indonesia Suicide Bombings & Criminal Cases Identification..... <i>Peter Sahelangi</i>	14
Vascular Anatomy and Its Clinical Application in Treatment Of Disease. Case Reports..... <i>Mohd Rizal Roslan</i>	15
<b>Kumpulan Materi Simposium Paralel</b>	
Sistem Pembelajaran Berbasis Teman Sebaya Sebagai Penunjang Pembelajaran Anatomi di Sistem Modul Mahasiswa FK Undip..... <i>Surya Pratama Brilliantika</i>	18
Variation of Hepatic Artery: A Clinical Review..... <i>Nikmatia Latief</i>	18
Human Endothelial Progenitor Cell: Biological Characteristics and Clinical Application.....	18

Evaluasi Variasi Arteri Renalis pada Pasien yang Dilakukan Pemeriksaan MDCT-Scan Angiografi Abdomen.....	19
<i>Rosdianah, Bachtiar Murtala, Nikmatia Latief</i>	
Aspek Anatomi Femur pada Penurunan Densitas Mineral Tulang.....	25
<i>Sitti Rafiah</i>	
Bone Growth .....	26
<i>Shelly Salmah</i>	
Rehabilitasi Fraktur Ekstremitas Bawah.....	27
<i>Yose Waluyo</i>	
Retina .....	29
<i>Batari Todja</i>	
Perubahan Anatomi dan Fisiologi pada Kasus Ablatio Retina .....	31
<i>A.M.Ichsan, Andi Pratiwi</i>	
Prefrontal Cortex: Basic to Clinic .....	40
<i>Taufik Pasiak</i>	
The Role of Mirror Neurons System in Mental Imagery Processing .....	45
<i>Muhammad Iqbal Basri</i>	
Clinical Neurophysiology in Evaluation of Cognitive Function.....	51
<i>Manfaluthy Hakim</i>	
<b>Kumpulan Materi Presentasi Oral</b>	
Hubungan Lompatan Jumping Smash dan Bentuk Arcus Pedis Dengan Kejadian Sprain Pergelangan Kaki pada Atlet Bulutangkis.....	54
<i>Rochman Basuki, Sigit Moerjono, Muhammad Arif Nurohman</i>	
Variasi Rugae Palatina pada Suku Jawa dan Batak.....	56
<i>Fidya, Iswara Rizki Arum Lestari, Diwya Nugrahini</i>	
A Study of Adult Human Cerebellum from The Anatomy Cadavers.....	61
<i>Kalanjati VP., Dewi AK., Santoso MWA</i>	
Identification of Sutural Bones in Indonesian Skulls .....	62
<i>Safrina D Ratnaningrum</i>	
Fleksibilitas Lumbal dan Hubungannya dengan Indeks Massa Tubuh pada Remaja Putra	64
<i>Tri Suciati</i>	
Gambaran Garis Lipat Tangan pada Anak Normal dan Anak Retardasi Mental di SLB Jakarta Pusat .....	64

<i>Etty Widayanti, Titiok Djannatun, Endang Purwaningsih, Restu Samsul Hadi, Mirfat</i>	
Peran Nitrit Oksida Intraluminal Pada Taut Gastroesofagus / <i>Gastroesophagus junction</i> (GEJ) .....	68
<i>Tena Djuartina, Ahmad Aulia, Ari Fahrial Syam</i>	
Pancreatic Beta Cells Protective Effect of Okra Extract in Alloxan-Induced Type II Diabetic Rats.....	70
<i>Karim Susanto, Anita Anjani</i>	
Kadar Hormon FSH Basal dan LH Basal Perempuan Sindrom Ovarium Polikistik (SOPK) Yang Mengikuti Program In Vitro Fertilization.....	74
<i>Roselina Panghiyangan, Dwi Anita Suryandari, Budi Wiweko, Mala Kurniati</i>	
Gambaran Histopatologi Hepar pada Tikus yang Diinfeksi <i>Plasmodium berghei</i> Pasca Pemberian Oksigen Hiperbarik .....	75
<i>Herin Setianingsih, Riami, Prawesty Diah Utami</i>	
Hepatocyte Expression of TNF- $\alpha$ in High Fat Diet Fed Wistar Rat with <i>Andrographis paniculata</i> Extract Therapy .....	80
<i>Hariadi, T.A.S., Jong, F.X.H.H</i>	
Theurapeutic Effects of <i>Andrographis Paniculata</i> Ethanol Extract in The Liver of Wistar Rat Treated with A High Fat Diet.....	88
<i>FX Himawan Haryanto Jong, Tania Ardiani Saleh Hariadi, Ari Gunawan, Mochamad Wirono Aman Santoso, Ni Wajan Tirthaningsih, Susilowati Andajani, Achmad Basori</i>	
Museum Anatomi Sebagai Salah Satu Sarana Pembelajaran Pendidikan Dokter.....	92
<i>Robilrawan dan Liliana Sugiharto</i>	
E-Forum in Anatomy Learning: Challenge and Advantages.....	95
<i>Lucky Prasetiowati, Sakina, Fundhy Sinar Ikrar Prihatanto</i>	
Process in Building E-Quiz Histology as An Innovative Assessment Model in Block Curriculum Faculty of Medicine Universitas Airlangga .....	95
<i>Rimbun, Tri Hartini Yuliawati, Dewi Ratna Sari, Kusuma Eko Purwantari, Anggun Raksyantoro</i>	
Student assistant in anatomy: A form of student engagement in the educational process ..	96
<i>Sakina, Fundhy Sinar Ikrar Prihatanto, Lucky Prasetiowati</i>	
Belajar Anatomi Traktus Substansia Alba Otak Menggunakan Media <i>Magnetic Resonancy Imaging Fiber Tractography</i> .....	96
<i>Herlina Uinarni</i>	
The Exposure Effect of Al-Qur'an Recitation to Spatial Memory on <i>Rattus novergicus</i> .....	99
<i>Abdurrahman Afa Haridhi, Nanang Wiyono, Yunia Hastami, Muthmainah</i>	
Penerapan Multivariate Adaptive Regresi Spline untuk Mengidentifikasi Faktor yang Berpengaruh pada Penggemukan Sapi di Desa Samaran Kabupaten Bojonegoro .....	99

Benjamin Christoffel Tehupuring dan Soeharsono

Inhibisi Sel Punca CD90 <sup>+</sup> Sumsum Tulang dan Sel Punca CD34 <sup>+</sup> Darah Tali Pusat Terhadap Aktivitas Fibrosis Sel Stelata Hepatik .....	100
<i>Radiana D Antarianto, Ahmad Aulia Jusuf, Atikah C Barasila, Wahyunia L Septiana, Ervina Julien Sitanggang</i>	
Signaling pathways involved in peripheral neuronal generation.....	101
<i>Ria Margiana, Jeanne Adiwinata Pawitan, Ahmad Aulia Jusuf</i>	
<i>Environmental Enrichment (EE) Mengurangi Perilaku Depresif Akibat Paparan Stres Kronis pada Tikus Putih (Rattus novvergicus) .....</i>	105
<i>Maia Thalia Giani, Muthmainah, Dyah Ratna Budiani, Nanang Wiyono</i>	
Concentration of Reactive Oxygen Species (ROS) and Telomeric Repeat Binding Factor2 (TERF2) On Individuals By Age and Gender.....	108
<i>Endang Purwaningsih, Tripanjiasih Susmiarsih, Yenni Zulhamidah, Achmad Sofwan</i>	
Mekanisme Kerja Obat Hipoglikemik Oral dan Hipoglikemik Herbal.....	115
<i>Yuliana</i>	
Evaluation Antacid Contain Magnesium Hidroxide Treatment Based on Parameter of Reactive Gastropathy .....	117
<i>Arni Kusuma Dewi, Chairul Anwar</i>	
Valproic Acid Reduces Pancreatic Beta Cells in Rat Offspring .....	117
<i>Ade Komariah, Bambang Kiranadi, Adi Winanto, Wasmen Manalu, Ekowati Handharyani</i>	
Evaluasi gambaran histologis penyembuhan luka bakar full thickness dengan pewarnaan Masson trichrome .....	118
<i>Dewi Sukmawati, Astheria Eryani, Elvin Clara Angmallsang, Lia Damayanti</i>	
Pengaruh Tipe Bentuk Jari Kaki, Model dan Ukuran Flat Shoes terhadap Kenyamanan Kaki pada Mahasiswi Fakultas Kedokteran Universitas Katolik Indonesia Atma Jaya .....	118
<i>Tan Fei Fan, Paula Chintya, Robi Irawan, Nawanto Agung Prastowo</i>	
Understanding The Anatomy of Chromosome and Its Abnormality: Role of An Anatomist ..	119
<i>Ni Wajan Tirthaningsih, Tri Hartini Yuliawati, Sakina, Lucky Prasetiowati</i>	
Kajian Pustaka: PPAR $\gamma$ agonis dan Sekresi Dopamin pada Kontrol Food Intake.....	120
<i>Fifi Veronica, Ronny Lesmana, Hanna G, Leonardo Lubis</i>	
Secang Wood Ethanol Extracts ( <i>Caesalpinia sappan</i> ) with potential effect on the quality of spermatozoa parameter of Wistar Strain Male Rats .....	122
<i>Andri Rezano, Nadiyah, Raden Ghita Sariwidyantry, Sunarjati Sugdoadi</i>	
Pengaruh Frekuensi Penggantian Balutan Kompres Betaine Polyhexanide 0,1% Terhadap Pembentukan Jaringan Epitel pada Luka Bakar Derajat II A Model Tikus Putih galur Wistar .....	129
<i>Rizky Oktavia Primasari, Heri Kristianto, Danik Agustin Purwantiningrum</i>	

Efek Kuratif Sari Kukusan Brokoli ( <i>Brassica oleracea L. var italica</i> ) Terhadap Kadar Nf-Kb Pada Mencit Model Kolitis .....	133
<i>Sijani Prahastuti, Jeanny Ervie Ladi, Teresa Liliana Wargasetia</i>	
Model MARS Pola Pertumbuhan Proventriculus, Ventriculus dan Intestinum tenue Ayam Pedaging Umur 1 hingga 4 minggu .....	135
<i>Siti F. Masruroh, B. C. Tehupuring, Surjo Kuncorojakti, Hana Eliyani, Suharsono</i>	
Pengaruh Pemberian Ekstrak Daun Teh Hijau Terhadap Konsentrasi dan Kecepatan Spermatozoa Tikus ( <i>Rattus norvegicus</i> ) Setelah Paparan Asap Rokok .....	138
<i>Tri Panjiasih Susmiarsih, Kenconoviyati, Kuslestari</i>	
Effect of Physical Stress on Cortisol Level and Diameter of White Pulp in Rats .....	143
<i>Tri Hartini Yuliawati, Rimbun, Viskasari P Kalanjati</i>	
<b>Kumpulan Materi Presentasi Poster</b>	
Transisi Epitel-Mesenkim dan Progres Kanker .....	150
<i>Oeij Anindita Adhika</i>	
Aktivitas Ekstrak Air Daun Kersen ( <i>Muntingia calabura</i> ) Terhadap Gambaran Histologis Sel Hepar <i>Mus Musculus Balb/C</i> yang Diinduksi D-Galaktosa .....	153
<i>Cempaka Jaga Paramudita, Dwi Nur Ahsani, Ika Fidianingsih, Evy Sulistyoningrum</i>	
Kajian literatur: Perbandingan Pengaruh Paparan Musik dan Bising Selama Periode Prenatal Terhadap Neurogenesis dan Sinaptogenesis Otak.....	154
<i>Fitri, Isabella Kurnia Liem</i>	
Effect of BCCAO Duration and Animal Models Sex on Brain Ischemic Volume After 24 Hours Reperfusion .....	165
<i>Ety S. Handayani, Titis Nurmasitoh, Saefudin Ali Akhmad, Afifah Nur Fauziah, Rizky Rizani, Rika Yulita Rahmawati, Angga Afriandi</i>	
Perbedaan Distribusi Tebal Lipatan Kulit antara Remaja Perempuan Madura di Sumenep dengan Surabaya .....	165
<i>Anung Putri Illahika, Abdurahman, Myrtati Dyah Artaria</i>	
Pengaruh Pemberian Propolis Terhadap Gambaran Histopatologi Gaster Tikus ( <i>Sprague dawley</i> ) yang Diberi Perlakuan Stres Isolasi Sosial .....	168
<i>Wiska Habiburohman Efendi, Kuswati, Zainuri Sabta Nugraha</i>	
Kajian Mikroskopis Ovarium dan Kompetensi Maturasi Oosit Domba dari Ovarium yang Disimpan Pada Suhu 4° C .....	168
<i>Masturi M</i>	
Comparison of The Crushing Point on Rat's Sciatic and Tibial Nerve for Peripheral Nerve Regeneration Study Model .....	169
<i>May Valzon, Ayu Permata</i>	

Pola Perkembangan Vertebra Cervicalis pada Mamalia ..... <i>Msy. Rulan Adnindya, Isabella Kumia Liem</i>	170
Effect of Ethanolic Extract of <i>Moringa oleifera</i> Leaves on Histopathology Image of Kidney <i>Subhan Amal Romis, Zainuri Sabta Nugraha, Kuswati</i>	170
The Effect of Caffeine Per Oral on The Neuron Cell Number and the Granuler Layer thickness of Dentate Gyrus, Formatio Hippocampalis..... <i>Kusuma Eko Purwanti, Tri Hartini Yulawati, Dewi Ratna Sari, Rimbun</i>	171
Korelasi Panjang Lengan Atas dan Panjang Sternum Perkutaneus dengan Tinggi Badan Mahasiswa Suku Banjar Fakultas Kedokteran Universitas Lambung Mangkurat..... <i>Iwan Afiane, Lena Rosida, Cynthia Pratiwi, Ramadhan Maulana Hikmat</i>	171
Anthropometric Indicators of Insulin Resistance on Obese Adults in Surabaya..... <i>Dewi Ratna Sari, Rimbun, Tri Hartini Yulawati, Kusuma Eko Purwanti</i>	175
Korelasi Nilai Ujian Praktikum Anatomi Dengan Indeks Prestasi Kumulatif Mahasiswa Fakultas Kedokteran Univesitas YARSI Angkatan 2013 ..... <i>Yenni Zulhamidah, Kenconoviyati, Achmad Sofwan, Kuslestari, Edward Syam, Aryenti, Zakiyah</i>	176
Profil Pasien Demam Berdarah dan Kadar Hemoglobin, Trombosit, dan Hematokrit pada Pasien Anak di RSUD Ratu Zalecha Martapura ..... <i>Ida Yuliana</i>	178
Hubungan antara Penggunaan Tas Sekolah dan Keluhan Muskuloskeletal pada Siswa Sekolah Dasar di Kecamatan Ilir Barat I Kota Palembang..... <i>Legiran, Tri Suciati, Meirisa Rahma Pratiwi</i>	181
Pengaruh Suplementasi Madu terhadap Migrasi dan Diferensiasi Sel Human Dermal Fibroblast (HDF) Sebagai Model Uji Luka In Vitro ..... <i>Restu Syamsul Hadi, Nadira, Yoan Rahmah Aprilia</i>	188
Disfungsi Kandung Kemih Non-Neurogenik pada Anak: Diagnosis dan Tata Laksana ..... <i>Bernadetha Nadeak</i>	192
Hubungan Paparan Gas Formaldehida di Ruang Praktikum Anatomi dengan Efek Iritasi Mata ..... <i>Sagala, Antonius Junedi, Margiana, Ria</i>	201
Air Pollution as A Cause Of Malignancy of Respiratory Organs in Riau Province..... <i>Laode Burhanuddin, Erizon, Delta Kurnia</i>	202
Factors that influence the academic performance of Malaysian Students in Faculty of Medicine Hasanuddin University ..... <i>Mohd. Azzam, Asty Amalia, St. Rafiah, M. Iqbal Basri</i>	205
Efek Kuratif Sari Kukusan Brokoli ( <i>Brassica oleracea L. var italica</i> ) Terhadap Gambaran Histopatologis Kolon Proksimal Dan Medial Pada Mencit Model Kolitis ..... <i>Mohd. Azzam, Asty Amalia, St. Rafiah, M. Iqbal Basri</i>	209

*Jeanny Ervie Ladi, Sijani Prahastuti, Teresa Liliana Wargasetia*

Gambaran Anatomi dan Histologi Saluran Pencernaan Ikan Bungo ( <i>Glossogobius Cf. aureus</i> ) dari Danau Tempe Sulawesi Selatan.....	209
<i>Satrya Adi Pratama, Wahyuni, I Ketut Mudite Adnyare, Andi Tamsil, Dwi Kesuma Sari</i>	
Hubungan Kadar Profil Lipid dengan Kejadian Penyakit Jantung Koroner di RSUP Dr. Wahidin Sudirohusodo Makassar .....	215
<i>Najdah Hidayah, A. Armyr Nurdin</i>	
Hubungan Status Menopause dengan Tingkat Depresi pada Wanita Usia 45-60 Tahun di Puskesmas Jumpang Baru Periode Juni 2016.....	215
<i>Reza Kurniawan Arta, Andi Alfian Zainuddin</i>	
<b>Kumpulan Materi Workshop</b>	
Immunohistochemistry: Principles and Methods .....	218
<i>Rahmawati Minhajat</i>	
Preparation Of Histological Slide for Light Microscopy .....	218
<i>Triani Hastuti Hatta</i>	
Item Development dan Item Review Soal MCQ Berbasis Kompetensi .....	219
<i>Sri Asriyani</i>	
Cognitive Assessment in Anatomy .....	220
<i>Irwin Aras</i>	

## **IMMUNOHISTOCHEMISTRY: PRINCIPLES AND METHODS**

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### **Abstract**

Immunohistochemistry (IHC) combines histological, immunological and biochemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label. IHC makes it possible to visualize the distribution and localization of specific cellular components within a cell or tissue.

The procedure of IHC consist of: tissue section, antigen retrieval, blocking endogenous enzyme, blocking background staining, primary antibody, secondary antibody, chromogen substrate, counterstain, mounting and microscopy observation.

## **I. Introduction**

Immunohistochemistry (IHC) combines histological, immunological and biochemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label. IHC makes it possible to visualize the distribution and localization of specific cellular components within a cell or tissue. This method especially useful for diagnosing abnormal cells in diseases such as cancer. In general, the information gained from IHC provides a valuable perspective that provide context for data obtained using other methods.

Immunohistochemical staining is accomplished with antibodies that recognize the target antigen. Since antibodies are highly specific, the antibody will bind only to the antigen of interest in the tissue section. The antibody-antigen interaction is then visualized using either chromogenic detection, in which an enzyme conjugated to the antibody catalyzes the conversion of a substrate to produce a colored precipitate at the location of the antigen, or fluorescent detection, in which a fluorophore is conjugated to the antibody and can be visualized using fluorescence microscopy.

Although immunohistochemistry (IHC) is a relatively straightforward experimental method, there are a number of variables that have to be optimized for each IHC study. Here, we summarize some of the variables that should be considered to ensure consistent and reproducible results.

## II. Principle of IHC

1. Primary antibody binds to specific antigen.
2. Antibody-antigen complex is bound by a secondary, enzyme conjugated, antibody.
3. In the presence of substrate and chromogen, the enzyme forms a colored deposit at the sites of antibody-antigen binding.
4. Materials (on glass Slide), Tissue (Paraffin block/frozen section)
5. Cellular antigen target in Cytoplasm, nuclear, cell membrane, lipids  
Proteins
6. Gives you a *spatial location (physically located)*
7. Can be used to locate particular cells, and proteins, identify different type of cells
8. Can be used to identify cellular events – e.g.apoptosis, proliferation

### Terminology

- Antigen: any molecule that has generated an antibody response. Epitope is part of antigen which react with antibody.
- Antibody: Immunoglobulin (mainly IgG) or glycoprotein that bind with high affinity and specificity to antigen.
  - Polyclonal antibody : are produced by different cells, Quicker and simpler to obtain by immunization of animal. More sensitivity but less specificity than monoclonal, cause heterogenitas nature of antibody.

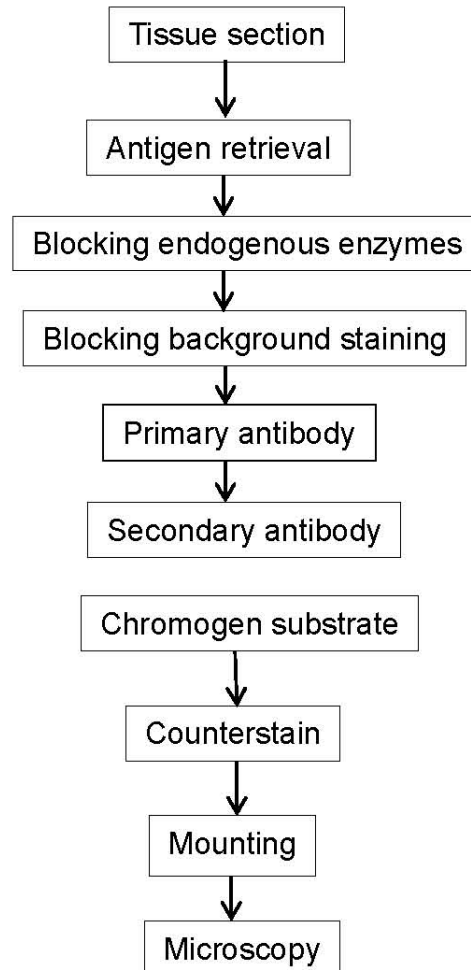
- Monoclonal antibody : the product of an individual clone of plasma cell, Monospecificity (to single epitope), Consuming time to generate but immortality

### **Important considerations for IHC**

- Antibody selection
- Fixation
- Sectioning
- Antigen Retrieval
- Blocking
- Controls (+ve &-ve)
- Direct method
- Indirect method
- Fluorescence
- Multiple labeling

It actually need to care about all this now because it may affect how you harvest your samples.

### III. Procedure of IHC



Sample preparation is key to producing high quality staining during immunohistochemistry (IHC). Sample preparation may include processes such as fixation, dehydration, embedding and sectioning. The two main methods of preserving tissues for IHC are paraffin embedding and freezing of the tissue (summarized below). The most appropriate route of sample preparation is usually determined by one or two experimental variables. For example, tissues may need to be snap-frozen if a phosphorylated epitope is being studied. Fixation of the tissue sample

is performed to preserve tissue morphology and retain the antigenicity of the target protein during the IHC experiment. The method of fixation often drives the design of the sample preparation workflow. Embedding following dehydration is often used both to preserve tissue morphology and to give the tissue support during sectioning. Additional steps in sample preparation for IHC may include antigen retrieval to unmask any epitopes that have been altered by fixation, permeabilization to grant the antibody access to intracellular proteins and blocking to prevent non-specific staining.

### **Key Stages in Preparing Histology Slides**

The five main stages in the preparation of histology slides are:

#### **1. Fixing:**

This process has two phases:

- 1) The coagulation or precipitation of the various components of the tissues and cells.
- 2) Their preservation in a state as nearly as possible like the living condition by forming stable chemical compounds.

The specimen is placed in a liquid fixing agent (chemical fixative) such as formaldehyde solution (formalin). This will slowly penetrate the tissue causing chemical and physical changes that will harden and preserve the tissue and protect it against subsequent processing steps. There are a limited number of reagents that can be used for fixation as they must possess particular properties that make them suitable for this purpose. Fixation time, generally this will mean that the specimen should fix for between

6 and 24 hours. \*The most common fixative for light microscopy is 10% neutral buffered formalin (4% formaldehyde in phosphate buffered saline).

## **2. Processing:**

Tissue processing is done to remove water from the biological tissues, replacing such water with a medium that solidifies, setting very hard and so allowing extremely thin sections to be sliced. This is important because biological tissue must be supported in an extremely hard solid matrix to enable sufficiently thin sections to be cut (5  $\mu\text{m}$  thick for light microscopy). For light microscopy, paraffin wax is most frequently used. Since it is immiscible with water, the main constituent of biological tissue, water must first be removed in the process of dehydration. Samples are transferred through baths of progressively more concentrated ethanol to remove the water. This is followed by a hydrophobic clearing agent (such as xylene) to remove the alcohol, and finally molten paraffin wax, the infiltration agent, which replaces the xylene.

## **3. Embedding:**

After the tissues have been dehydrated, cleared, and infiltrated with the embedding material, they are ready for external embedding. During this process the tissue samples are placed into molds along with liquid embedding material (Paraffin wax) which is then hardened by cooling. The hardened blocks containing the tissue samples are then ready to be sectioned. Because Formalin-fixed, paraffin-embedded tissues may be stored indefinitely at room temperature, and nucleic acids (both DNA

and RNA) may be recovered from them decades after fixation. Formalin-fixed, paraffin-embedded tissues are an important resource for historical studies in medicine.

#### **4. Sectioning**

For light microscopy, a steel knife mounted in a microtome is used to cut (4 $\mu$ m - 10 $\mu$ m)-thick tissue sections which are mounted on a glass microscope slide. Then the mounted sections are treated with the appropriate stain. Sections can be cut through the tissue in a number of directions.

Possible orientations at which tissue samples may be sectioned include:

- Vertical sectioning perpendicular (at right-angles) to the surface of the tissue. This is the most common method.
- Horizontal sectioning is often done for the study of hair follicles and structures that include hairs, hair follicles, arrector pili muscles, and sebaceous glands in general. Such structures are sometimes called "pilosebaceous units".
- Tangential to horizontal sectioning is done in chemosurgery (also called "Mohs surgery") which is a form of microscopically controlled surgery used to treat certain types of skin cancer.

#### **5. Staining:**

Finally, the mounted sections are treated with an appropriate histology stain. Biological tissue has little inherent contrast in either the light or electron microscope. Staining is employed to give contrast to the tissue as well as highlighting particular features of interest. Where the underlying mechanistic chemistry of staining is

understood, the term histochemistry is used. Haematoxyline and eosin (H&E stain) is the most commonly used light microscopical stain in histology and histopathology. Haematoxyline, a basic dye, stains nuclei blue due to an affinity to nucleic acids in the cell nucleus; eosin, an acidic dye, stains the cytoplasm pink.

### Common laboratory stains

Stain	Common use	Nucleus	Cytoplasm	Red blood cell (RBC)	Collagen fibers	Specifically stains
<b>Haematoxyline</b>	General staining when paired with eosin (i.e. H&E)	Orange, Cyan Blue or Green	Blue/Brown/Black	N/A	N/A	Nucleic acids—blue (endoplasmic reticulum)—blue
<b>Eosin</b>	General staining when paired with Haematoxyline (i.e. H&E)	N/A	Pink	Orange/red	Pink	Elastic fibers—pink Collagen fibers—pink Reticular fibers—pink
<b>Toluidine blue</b>	General staining	Blue	Blue	Blue		Mast cells granules—purple
<b>Masson's trichrome stain</b>	Connective tissue	Black	Red/pink	Red	Blue/green	Cartilage—blue/green Muscle fibers—red
<b>Mallory's trichrome stain</b>	Connective tissue	Red	Pale red	Orange	Deep blue	Keratin—orange Cartilage—blue Bone matrix—deep blue Muscle fibers—red
<b>Weigert's elastic stain</b>	Elastic fibers	Blue/black	N/A	N/A	N/A	Elastic fibers—blue/black
<b>Heidenhain's AZAN trichrome stain</b>	Distinguishing cells from extracellular components	Red/purple	Pink	Red	Blue	Muscle fibers—red Cartilage—blue Bone matrix—blue
<b>Silver staining</b>	Reticular fibers, nerve fibers, fungi	N/A	N/A	N/A	N/A	Reticular fibers—brown/black Nerve fibers—brown/black Fungi—black
<b>Wright's stain</b>	Blood cells	Bluish/purple	Bluish/gray	Red/pink	N/A	Neutrophil granules—purple/pink Eosinophil granules—bright red/orange Basophil granules—deep purple/violet Platelet granules—red/purple
<b>Orcein stain</b>	Elastic fibers	Deep blue	N/A	Bright red	Pink	Elastic fibers—dark brown Mast cells

						granules—purple Smooth muscle— light blue
<b>Periodic acid– Schiff(PAS)</b>	Basement membrane, localizing carbohydrates	Blue	N/A	N/A	Pink	Glycogen and other carbohydrates— magenta

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#### **IV. Application of Immunohistochemistry**

Since IHC involves specific antigen–antibody reactions, it has apparent advantage over traditionally used special enzyme staining techniques that identify only a limited number of proteins, enzymes, and tissue structures. Therefore, IHC has become a crucial technique and is widely used in many medical research laboratories as well as clinical diagnostics of disease and tumors of uncertain histogenesis, prognostic markers in cancer, prediction of response to therapy, determine the function of specific gene products and research application.

#### **Prognostic markers in cancer**

To predict the prognosis of tumors by identification of enzymes, tumor-specific antigens, oncogenes, tumor suppressor genes, and tumor cell proliferation markers. Analysis of tumors by these methods is a significant improvement over the conventional prognostic considerations by clinical staging and histologic grading. IHC is used for disease diagnosis, drug development, and biological research. Using specific tumor markers, physicians use IHC to diagnose a cancer as benign or malignant, determine the stage and grade of a tumor, and identify the cell type and origin of a metastasis to find the site of the primary tumor. IHC is also used in drug development to test drug efficacy by detecting either the activity or the up- or down-regulation of disease targets.

### **Tumors of uncertain histogenesis**

IHC methods have brought about a revolution in approach to diagnosis of tumors of uncertain origin, primary as well as metastatic from unknown primary tumor. A panel of antibodies is chosen to resolve such diagnostic problem cases. The selection of antibodies being made is based on clinical history, morphological features, and results of other relevant investigations. Immunohistochemical stains for intermediate filaments are expressed by tumor cells (keratin, desmin, vimentin, neurofilaments, and glial fibrillary acidic proteins).

### **Prediction of response to therapy**

Immunohistochemical methods are also being applied to confirm infectious agent in tissues by use of specific antibodies against microbial DNA or RNA, e.g. in Cytomegalo virus, Hepatitis B virus, Hepatitis C virus, etc. The application is used routinely in validation of disease targets as it allows visualizing expression of the target in the affected tissue during the disease process. The concept was introduced as early as the 1940s when fluorescein dye (visible under ultraviolet light) was tagged to antibodies directed against pneumococci for identification of this organism with specific anti-serum.<sup>[7]</sup> This method, often abbreviated IFA for “immunofluorescence assay”, has been widely used for the detection of specific pathogens, viral as well as bacterial and protozoal, in “fresh”/unfixed tissues in both human and veterinary medicine.

Another important advantage of IHC is that it can also be used to detect organisms in cytological preparations such as fluids, sputum samples, and material obtained from fine needle aspiration procedures. This can be very helpful in certain

situations such as detection of pneumocystis from the sputum of an immunocompromised patient who needs rapid and precise confirmation of infection in order to begin immediate and appropriate therapy.

### **In Genetics**

Immunohistochemistry can also be used to determine the function of specific gene products in fundamental biological processes such as development and apoptosis. Using a custom made monoclonal antibody against p53 homologue of the pro-apoptotic pathways of p53 was identified.

### **Research application**

Much of the current research into the causes of neurodegenerative diseases is directed at identifying the factors that result in the formation of paired helical filaments, the deposition of beta amyloid, cytoplasmic accumulations of alpha synuclein, etc. Consequently, studies to localize and quantify the abnormal proteins that constitute reasons of neurodegenerative diseases are of central importance. IHC using antibodies to beta amyloid, alpha synuclein, ubiquitin, huntingtin, polyglutamine, and others has become a routine tool for a sensitive detection and quantification of these abnormal proteins in both human tissues and in experimental animals that are used to model some of the features of these diseases. IHC is an important tool in diagnostic and research laboratories.

## **V. Summary**

Immunohistochemistry (IHC) combines histological, immunological and biochemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label. The principles of IHC are primary antibody binds to specific antigen, antibody-antigen complex is bound by a secondary, enzyme conjugated, antibody In the presence of substrate and chromogen, the enzyme forms a colored deposit at the sites of antibody-antigen binding.

Therefore, IHC has become a crucial technique and is widely used in many medical research laboratories as well as clinical diagnostics of disease and tumors of uncertain histogenesis, prognostic markers in cancer, prediction of response to therapy, determine the function of specific gene products and research application.

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