

# Methods in ENZYMOLOGY

Volume 534

Endosome Signaling  
Part A

*Edited by*

P. Michael Conn





VOLUME FIVE HUNDRED AND THIRTY FOUR

# METHODS IN ENZYMOLGY

## Endosome Signaling Part A

Edited by

**P. MICHAEL CONN**

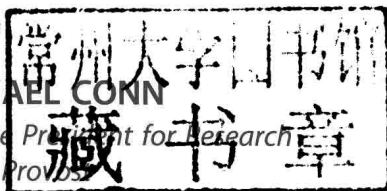
*Senior Vice President for Research*

*Associate Provost*

*Professor of Internal Medicine and Cell Biology*

*Texas Tech University Health Sciences Center*

*Lubbock, TX 79430, USA*



AMSTERDAM • BOSTON • HEIDELBERG • LONDON  
NEW YORK • OXFORD • PARIS • SAN DIEGO  
SAN FRANCISCO • SINGAPORE • SYDNEY • TOKYO

Academic Press is an imprint of Elsevier



Academic Press is an imprint of Elsevier  
525 B Street, Suite 1800, San Diego, CA 92101-4495, USA  
225 Wyman Street, Waltham, MA 02451, USA  
Radarweg 29, PO Box 211, 1000 AE Amsterdam, The Netherlands  
The Boulevard, Langford Lane, Kidlington, Oxford, OX5 1GB, UK  
32 Jamestown Road, London NW1 7BY, UK

First edition 2014

Copyright © 2014 Elsevier Inc. All Rights Reserved.

No part of this publication may be reproduced, stored in a retrieval system or transmitted in any form or by any means electronic, mechanical, photocopying, recording or otherwise without the prior written permission of the publisher

Permissions may be sought directly from Elsevier's Science & Technology Rights Department in Oxford, UK: phone (+44) (0) 1865 843830; fax (+44) (0) 1865 853333; email: [permissions@elsevier.com](mailto:permissions@elsevier.com). Alternatively you can submit your request online by visiting the Elsevier web site at <http://elsevier.com/locate/permissions>, and selecting *Obtaining permission to use Elsevier material*

#### Notice

No responsibility is assumed by the publisher for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions or ideas contained in the material herein. Because of rapid advances in the medical sciences, in particular, independent verification of diagnoses and drug dosages should be made

For information on all Academic Press publications  
visit our website at [store.elsevier.com](http://store.elsevier.com)

ISBN: 978-0-12-397926-1

ISSN: 0076-6879

Printed and bound in United States of America

14 15 16 17 11 10 9 8 7 6 5 4 3 2 1



Working together  
to grow libraries in  
developing countries

[www.elsevier.com](http://www.elsevier.com) • [www.bookaid.org](http://www.bookaid.org)



VOLUME FIVE HUNDRED AND THIRTY FOUR

# **METHODS IN ENZYMOLGY**

Endosome Signaling Part A

# METHODS IN ENZYMOLOGY

*Editors-in-Chief*

**JOHN N. ABELSON and MELVIN I. SIMON**

*Division of Biology  
California Institute of Technology  
Pasadena, California*

**ANNA MARIE PYLE**

*Departments of Molecular, Cellular and Developmental  
Biology and Department of Chemistry  
Investigator, Howard Hughes Medical Institute  
Yale University*

**GREGORY L. VERDINE**

*Department of Chemistry and Chemical Biology  
Harvard University*

*Founding Editors*

**SIDNEY P. COLOWICK and NATHAN O. KAPLAN**

# CONTRIBUTORS

**Mihaela Anitei**

Biotechnology Center, Dresden University of Technology, Dresden, Germany

**Spyros Artavanis-Tsakonas**

Department of Cell Biology, Harvard Medical School, Boston, Massachusetts, USA

**Yoriko Atomi**

Graduate School of Information Science and Technology, and Radioisotope Center, Cell to Body Dynamics Laboratory 1, The University of Tokyo, Tokyo, Japan

**Nicolas F. Berbari**

Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, Alabama, USA

**Leanne Bilawchuk**

Li Ka Shing Institute of Virology, Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada

**Johanne Bouthillier**

Centre de recherche en rhumatologie et immunologie, Centre Hospitalier Universitaire de Québec, Québec, Canada

**Andreas Brech**

Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, and Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway

**Sophia Y. Breusegem**

Department of Clinical Biochemistry, Cambridge Institute for Medical Research, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom

**Jorge Cancino**

Department of Life Sciences, Institute of Protein Biochemistry, National Research Council and Telethon Institute of Genetics and Medicine, Naples, Italy

**C. Yan Cheng**

The Mary M. Wohlford Laboratory for Male Contraceptive Research, Center for Biomedical Research, Population Council, New York, USA

**Brett M. Collins**

Institute for Molecular Bioscience, University of Queensland, St. Lucia, Queensland, Australia

**Emmanuel Culetto**

Centre de Génétique Moléculaire, CNRS UPR3404, Université Paris-Sud, 91198 Gif-sur-Yvette Cedex, France

**Manlio Di Cristina**

Department of Experimental Medicine and Biochemical Sciences, Section of Biochemistry and Molecular Biology, University of Perugia, Perugia, Italy

**Carla Emiliani**

Department of Experimental Medicine and Biochemical Sciences, Section of Biochemistry and Molecular Biology, University of Perugia, Perugia, Italy

**Mitsunori Fukuda**

Laboratory of Membrane Trafficking Mechanisms, Department of Developmental Biology and Neurosciences, Graduate School of Life Sciences, Tohoku University, Sendai, Miyagi, Japan

**Emilia Galperin**

Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, Kentucky, USA

**Rajesh Ghai**

Institute for Molecular Bioscience, University of Queensland, St. Lucia, Queensland, Australia

**Monica Giannotta**

FIRC Institute of Molecular Oncology Foundation, Milan, Italy

**Jennifer M. Gillette**

Department of Pathology, University of New Mexico Health Sciences Center, Albuquerque, New Mexico, USA

**Marcos González-Gaitán**

Department of Biochemistry, Faculty of Sciences, and Department of Molecular Biology, Faculty of Sciences, Geneva University, Geneva, Switzerland

**Richard G. Hegele**

Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

**Bernard Hoflack**

Biotechnology Center, Dresden University of Technology, Dresden, Germany

**Kazuya Hori**

Department of Cell Biology, Harvard Medical School, Boston, Massachusetts, USA

**Myoungkun Jeoung**

Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, Kentucky, USA

**Taiho Kambe**

Graduate School of Biostudies, Kyoto University, Kyoto, Japan

**Christophe Lefebvre**

Centre de Génétique Moléculaire, CNRS UPR3404, Université Paris-Sud, 91198 Gif-sur-Yvette Cedex, France

**Renaud Legouis**

Centre de Génétique Moléculaire, CNRS UPR3404, Université Paris-Sud, 91198 Gif-sur-Yvette Cedex, France

**Guy M. Lenk**

Department of Human Genetics, University of Michigan, Ann Arbor, Michigan, USA

**Pearl P.Y. Lie**

The Mary M. Wohlford Laboratory for Male Contraceptive Research, Center for Biomedical Research, Population Council, New York, USA

**Sylvain Loubéry**

Department of Biochemistry, Faculty of Sciences, and Department of Molecular Biology, Faculty of Sciences, Geneva University, Geneva, Switzerland

**Alessandro Magini**

Department of Experimental Medicine and Biochemical Sciences, Section of Biochemistry and Molecular Biology, University of Perugia, Perugia, Italy

**Marion Manil-Ségalen**

Centre de Génétique Moléculaire, CNRS UPR3404, Université Paris-Sud, 91198 Gif-sur-Yvette Cedex, France

**Roberta Mannucci**

IMAGE Analysis Laboratory, University of Perugia, Perugia, Italy

**François Marceau**

Centre de recherche en rhumatologie et immunologie, Centre Hospitalier Universitaire de Québec, Québec, Canada

**David J. Marchant**

Li Ka Shing Institute of Virology, Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada

**Kristopher D. Marjon**

Department of Pathology, University of New Mexico Health Sciences Center, Albuquerque, New Mexico, USA

**Takahide Matsui**

Laboratory of Membrane Trafficking Mechanisms, Department of Developmental Biology and Neurosciences, Graduate School of Life Sciences, Tohoku University, Sendai, Miyagi, Japan

**Miriam H. Meisler**

Department of Human Genetics, University of Michigan, Ann Arbor, Michigan, USA

**Mehdi Mobli**

Centre for Advanced Imaging & School of Chemistry and Molecular Biosciences, The University of Queensland, St. Lucia, Queensland, Australia

**Dolores D. Mruk**

The Mary M. Wohlford Laboratory for Male Contraceptive Research, Center for Biomedical Research, Population Council, New York, USA

**Ildo Nicoletti**

IMAGE Analysis Laboratory, University of Perugia, Perugia, Italy

**Christian Niehage**

Biotechnology Center, Dresden University of Technology, Dresden, Germany



**Gordon Nish**

Li Ka Shing Institute of Virology, Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada

**Asami Oguro-Ando**

Department of Neuroscience and Pharmacology, Rudolf Magnus Institute of Neuroscience, University Medical Centre Utrecht, Utrecht, The Netherlands

**Eri Ohoto-Fujita**

Graduate School of Information Science and Technology, and Radioisotope Center, Cell to Body Dynamics Laboratory 1, The University of Tokyo, Tokyo, Japan

**Alice Polchi**

Department of Experimental Medicine and Biochemical Sciences, Section of Biochemistry and Molecular Biology, University of Perugia, Perugia, Italy

**Caroline Roy**

Centre de recherche en rhumatologie et immunologie, Centre Hospitalier Universitaire de Québec, Québec, Canada

**Carmen Ruggiero**

Department of Cellular and Translational Pharmacology, Fondazione Mario Negri Sud, Unit of Genomic Approaches to Membrane Traffic, Santa Maria Imbaro (CH), Italy

**Michele Sallese**

Department of Cellular and Translational Pharmacology, Fondazione Mario Negri Sud, Unit of Genomic Approaches to Membrane Traffic, Santa Maria Imbaro (CH), Italy

**Kay Oliver Schink**

Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, and Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway

**Matthew N.J. Seaman**

Department of Clinical Biochemistry, Cambridge Institute for Medical Research, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom

**Anindya Sen**

Department of Cell Biology, Harvard Medical School, Boston, Massachusetts, USA

**Miho Shimizu**

Graduate School of Information Science and Technology, and Radioisotope Center, Cell to Body Dynamics Laboratory 1, The University of Tokyo, Tokyo, Japan

**Christoph Stange**

Biotechnology Center, Dresden University of Technology, Dresden, Germany

**Harald Stenmark**

Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, and Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway

**Melanie L. Styers**

Department of Biology, Birmingham-Southern College, Birmingham, Alabama, USA

**Elizabeth Sztul**

Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, Alabama, USA

**Brunella Tancini**

Department of Experimental Medicine and Biochemical Sciences, Section of Biochemistry and Molecular Biology, University of Perugia, Perugia, Italy

**Cristy Tower-Gilchrist**

Department of Cell Biology, Emory University, Atlanta, Georgia, USA

**Lorena Urbanelli**

Department of Experimental Medicine and Biochemical Sciences, Section of Biochemistry and Molecular Biology, University of Perugia, Perugia, Italy

**Catherine Sem Wegner**

Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, and Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway

**Chris K.C. Wong**

Department of Biology, Hong Kong Baptist University, Hong Kong, China

**Elissa W.P. Wong**

The Mary M. Wohlford Laboratory for Male Contraceptive Research, Center for Biomedical Research, Population Council, New York, USA

**Xiang Xiao**

The Mary M. Wohlford Laboratory for Male Contraceptive Research, Center for Biomedical Research, Population Council, New York, USA, and Department of Reproductive Physiology, Zhejiang Academy of Medical Sciences, Hangzhou, Zhejiang, China

**Bradley K. Yoder**

Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, Alabama, USA

## PREFACE

Endosomes are membrane-bound compartments that transport internalized material from the plasma membrane to the lysosome and elsewhere. These compartments, often about 500 nm, but ranging in size, have the capability to sort molecules, routing some contents to the lysosomes for degradation, and recycling other materials back to the plasma membrane. The Golgi apparatus also provides molecules to the endosome, some of which are delivered to lysosomes and others are recycled back to the Golgi. Because of this ability to differentially deliver molecules, the endosome is viewed as a presorting structure.

Endosomes are categorized by size, enzymatic content, morphology, and by other criteria such as the length of time it takes internalized material to reach them. Endosomes may provide platforms for cross talk between signaling systems, and this consideration has provided them elite status among cellular components that contribute to signaling.

This volume provides descriptions of the range of methods used to analyze and evaluate these important compartments. The authors explain how these methods are able to provide important biological insights in the context of particular models.

Authors were selected based on both their research contributions and on their ability to describe their methodological contributions in a clear and reproducible way. They have been encouraged to make use of graphics, comparisons to other methods, and to provide tricks and approaches not revealed in prior publications that make it possible to adapt their methods to other systems.

The editor wants to express appreciation to the contributors for providing their contributions in a timely fashion, to the senior editors for guidance, and to the staff at Academic Press for helpful input.

P. MICHAEL CONN  
Lubbock, TX, USA

# CONTENTS

<i>Contributors</i>	<i>xiii</i>
<i>Preface</i>	<i>xix</i>

## Section I Compartments

<b>1. Monitoring Phosphatidylinositol 3-Phosphate in Multivesicular Endosome Biogenesis</b>	<b>3</b>
Catherine Sem Wegner, Kay Oliver Schink, Harald Stenmark, and Andreas Brech	
1. Introduction	4
2. Localization of PtdIns3P and EGFR	5
3. Correlative Light and Electron Microscopy of MVEs	16
4. Concluding Remarks	21
Acknowledgments	22
References	22
<b>2. Methods to Discriminate the Distribution of Acidic Glycohydrolases Between the Endosomal–Lysosomal Systems and the Plasma Membrane</b>	<b>25</b>
Alessandro Magini, Alice Polchi, Brunella Tancini, Lorena Urbanelli, Manlio Di Cristina, Roberta Mannucci, Ildo Nicoletti, and Carla Emiliani	
1. Introduction	27
2. Purification of Lipid Microdomains from Cell Membranes and Glycohydrolases Activity Determination	28
3. Discrimination of Cell Surface Lipid Microdomain-Associated Glycohydrolases from the Intracellular Counterparts	31
4. Immunology Capture of Lipid Microdomains Containing Glycohydrolases	35
5. <i>In Vivo</i> Assay of Cell Surface Glycohydrolases	35
6. Fluorescence Microscopy Analysis of Hex Intracellular Trafficking	38
7. Summary	43
Acknowledgment	44
References	44

<b>3. Visualizing of Signaling Proteins on Endosomes Utilizing Knockdown and Reconstitution Approach</b>	<b>47</b>
Myoungkun Jeoung and Emilia Galperin	
1. Introduction	48
2. Description of Methods	49
3. Conclusions	61
Acknowledgments	62
References	62
<b>4. Virus-Induced Signaling Influences Endosome Trafficking, Virus Entry, and Replication</b>	<b>65</b>
David J. Marchant, Leanne Bilawchuk, Gordon Nish, and Richard G. Hegele	
1. Introduction	66
2. Isolation of Fibroblasts from Wild-Type or MyD88 <sup>-/-</sup> Mice and Purification of Virus	66
3. Screening Assays for the Determination of Principal Host Kinases Involved During Virus Replication	68
4. Virus-Host Cell Receptor Interactions and Kinase Activation	70
5. Detection of Virus-Induced Cell Signaling and Virus Entry into Endosomes Using Immunofluorescence Confocal Microscopy	73
6. Summary	75
References	75
<b>5. Methods to Evaluate Zinc Transport into and out of the Secretory and Endosomal-Lysosomal Compartments in DT40 Cells</b>	<b>77</b>
Taiho Kambe	
1. Introduction	78
2. Intracellular Zinc Transporters Localized to the Secretory and Endosomal-Lysosomal Compartments Play Crucial Roles	79
3. Establishment of DT40 Cells Deficient in Zinc Transporters Genes	82
4. Experimental Procedures Used in Studies of DT40 Cells Deficient in Zinc Transport	84
5. Functional Analysis of Zinc Mobilization into or out of the Secretory and Endosomal-Lysosomal Compartments	86
6. Concluding Remarks	89
Acknowledgment	89
References	89

<b>6. Interactions Between Endosomal Maturation and Autophagy: Analysis of ESCRT Machinery During <i>Caenorhabditis elegans</i> Development</b>	<b>93</b>
Marion Manil-Ségalen, Emmanuel Culetto, Renaud Legouis, and Christophe Lefebvre	
1. Introduction	94
2. Strains and Reagents	95
3. Fluorescent-Tagged Protein Construction and Transgenesis	98
4. Analysis of Developmental Phenotypes in ESCRT Mutants	100
5. Analysis of Vesicular Compartments	104
6. Analysis of Autophagy	110
7. Methods to Visualize Amphisome, the Fusion Organelle Between Endosomes and Autophagosomes	114
8. Conclusions	116
Acknowledgments	116
References	116
<b>7. Assessment of Cation Trapping by Cellular Acidic Compartments</b>	<b>119</b>
François Marceau, Caroline Roy, and Johanne Bouthillier	
1. Introduction	120
2. Quinacrine Uptake by Cells	123
3. Macroautophagic Accumulation in Cells That Have Accumulated Cations	128
4. Summary	129
Acknowledgments	130
References	130
<b>8. Signaling Initiated by the Secretory Compartment</b>	<b>133</b>
Carmen Ruggiero, Jorge Cancino, Monica Giannotta, and Michele Sallèse	
1. Introduction	134
2. ER-to-Golgi Traffic-Synchronization Protocols	136
3. Read-Outs for Traffic-Generated Signaling	141
4. KDEL <sub>R</sub> Signaling	147
5. The KDEL <sub>R</sub> Transduction Machinery	149
6. Conclusions	151
Acknowledgments	151
References	152

<b>9. Image-Based and Biochemical Assays to Investigate Endosomal Protein Sorting</b>	<b>155</b>
Sophia Y. Breusegem and Matthew N.J. Seaman	
1. Introduction	156
2. Antibody-Uptake Assays	157
3. Detailed Characterization of Endosomes	169
4. Endosome Recruitment and/or Association	171
5. Summary	177
Acknowledgments	177
References	177

## Section II

### Transport and Transfer

<b>10. Cytokines, Polarity Proteins, and Endosomal Protein Trafficking and Signaling—The Sertoli Cell Blood–Testis Barrier System <i>In Vitro</i> as a Study Model</b>	<b>181</b>
Xiang Xiao, Elissa W.P. Wong, Pearl P.Y. Lie, Dolores D. Mruk, Chris K.C. Wong, and C. Yan Cheng	
1. Introduction	182
2. Endocytosis Assay	184
3. Materials	187
4. Buffers	187
5. Methods	188
6. Cell Staining to Assess Endocytosis	190
7. Results	190
8. Summary	191
Acknowledgments	192
References	192
<b>11. Methods of Analysis of the Membrane Trafficking Pathway from Recycling Endosomes to Lysosomes</b>	<b>195</b>
Takahide Matsui and Mitsunori Fukuda	
1. Introduction	196
2. Degradation of TfR in Lysosomes	197
3. Screening Methods for Rab Proteins Involved in Lysosomal Degradation of TfR	200
4. Effect of Rab12 Knockdown on an EGFR Endocytic Pathway and a Tf Recycling Pathway	203

5. Concluding Remarks	205
Acknowledgments	205
References	205
<b>12. Measurement of Intercellular Transfer to Signaling Endosomes</b>	<b>207</b>
Kristopher D. Marjon and Jennifer M. Gillette	
1. Introduction	208
2. Labeling of Transferred Signals	209
3. Measuring ICT Using Fluorescence Techniques	214
4. Signaling Endosomes	216
5. Experimental Example: ICT to SARA-Positive Signaling Endosomes	217
6. Summary	218
References	219
<b>13. Liposome-Based Assays to Study Membrane-Associated Protein Networks</b>	<b>223</b>
Christian Niehage, Christoph Stange, Mihaela Anitei, and Bernard Hoflack	
1. Introduction	224
2. Isolation of Core Machineries Required for Carrier Biogenesis on Synthetic Membranes	226
3. Identification of Core Machineries by Mass Spectrometry-Based, Label-Free Quantitative Proteomics	234
4. Visualization of Protein Dynamics on Giant Unilamellar Vesicles by Fluorescence Microscopy	238
5. Summary	241
Acknowledgments	242
References	242
<b>14. Mouse Models of PI(3,5)P<sub>2</sub> Deficiency with Impaired Lysosome Function</b>	<b>245</b>
Guy M. Lenk and Miriam H. Meisler	
1. Introduction	246
2. Design of Mouse Models	247
3. A Spontaneous Null Mutation of <i>Fig4</i> : The <i>Pale Tremor</i> Mouse	249
4. Tissue-Specific <i>Fig4</i> Transgenes: Neurons Versus Astrocytes	251
5. Conditional Knockout of <i>Fig4</i> in Neurons	251
6. The Human Disease Mutation FIG4-I41T in Transgenic Mice	252
7. A Spontaneous Missense Mutation of <i>Vac14</i> in the <i>ingls</i> Mouse	252
8. A Null Gene-Trap Allele of <i>Vac14</i>	253



9. A Hypomorphic Gene-Trap Allele of <i>Pikfyve</i> ( <i>Fab1</i> )	253
10. A Conditional Knockout of <i>Pikfyve</i>	254
11. Genetic Interactions: <i>Fig4</i> , <i>Vac14</i> , and <i>Mtmr2</i>	254
12. Genetic Effects of Strain Background	255
13. Future Applications of Mouse Models of PI(3,5)P <sub>2</sub> Deficiency	255
Acknowledgments	257
References	257
<b>15. Monitoring Endosomal Trafficking of the G Protein-Coupled Receptor Somatostatin Receptor 3</b>	<b>261</b>
Cristy Tower-Gilchrist, Melanie L. Styers, Bradley K. Yoder, Nicolas F. Barbari, and Elizabeth Sztul	
1. Introduction	262
2. Development of Cell Lines Stably Expressing SSTR3	264
3. Live Imaging of SSTR3 and RABS in Mammalian Kidney Cells	269
4. Dynamics of SSTR3 Transit Relative to RABS	272
5. Effects of Dominant Negative Rabs on SSTR3 Trafficking	276
6. Summary	278
Acknowledgments	278
References	278
<b>Section III</b>	
<b>Proteins</b>	
<b>16. Genetic Circuitry Modulating Notch Signals Through Endosomal Trafficking</b>	<b>283</b>
Kazuya Hori, Anindya Sen, and Spyros Artavanis-Tsakonas	
1. Introduction	284
2. Genetic Screen Using the Exelixis Collection	285
3. Notch Localization in Endosomes	289
4. Optical Approaches	292
5. Ubiquitination Status of Notch	295
6. Conclusion	297
Acknowledgments	297
References	297
<b>17. Monitoring Notch/Delta Endosomal Trafficking and Signaling in <i>Drosophila</i></b>	<b>301</b>
Sylvain Loubéry and Marcos González-Gaitán	
1. Introduction	302
2. Antibody Uptake Assays to Monitor Notch and Delta Trafficking	305