Phone: 49-(89)-8578-2525

Fax: 49-(89)-8578-3777

(h) Measurement of NADPH Oxidase Activity with Hydroethidine

Submitted by:

Gregor Rothe and Günter Valet Arbeitsgruppe Zellbiochemie Max-Planck-Institut für Biochemie W-8033 Martinsried Germany

Outline

During the oxidative burst, phagocytic cells release superoxide anion through the membrane-bound NADPH oxidase. Hydrogen peroxide, produced by dismutation of superoxide anion, is the substrate for the myeloperoxidase-catalyzed oxidation inside the phagosome. Hydroethidine can be oxidized to red fluorescent ethidium bromide by superoxide anion directly, in addition to oxidation by hydrogen peroxide and peroxidase. This is in contrast to substrates such as dihydrorhodamine 123 or 2',7'dichlorofluorescin which are not oxidized by the superoxide anion. Stimulation of neutrophils with PMA or by phagocytosis of bacteria results in an 8- to 20-fold increase of cellular red fluorescence.

Specimen: 3 ml heparinized human blood (10 U heparin/ml)

Reagents

HBSS without phenol red or bicarbonate, supplemented with 10 mM HEPES (pH 7.35)

hydroethidine (MW 315)

stock solution: 10 mM in DMF (3.15 mg/ml)

working solution: 1 mM (1:10 dilution of stock in HBSS)

propidium iodide (PI) (MW 668.4)

stock solution: 3 mM (2 mg/ml) in 5 mM HEPES-buffered saline (0.15 M NaCl, pH 7.35)

trypan blue (MW 960.8)

stock solution: 10 mM in 5 mM HEPES-buffered saline (0.15 M NaCl, pH 7.35)

phorbol 12-myristate 13-acetate (PMA) (MW 616.8)

stock solution: 1 mM in DMF

working solution: 10 μ M (1:100 dilution of stock in HBSS)

Procedure

- 1. Layer 3 ml heparinized blood carefully on top of 3 ml lymphocyte separation medium. Allow erythrocytes to sediment for 40 minutes at room temperature.
- 2. Withdraw the upper $800\,\mu l$ supernatant plasma and store on ice. This will contain platelets and approximately 2 x 107/ml unseparated leukocytes.
- 3. For PMA stimulation, put 1.00 ml HBSS, 20 µl cell suspension, and 10 µl hydroethidine working solution in a 12 x 75 mm polypropylene test tube (final hydroethidine concentration 10 μ M). Incubate for 5 minutes at 37°C. Add 10 μ l PMA working solution (final PMA concentration 100 nM). Continue incubation, taking 250 µl aliquots at 10, 20, and 30 minutes after addition.
- 4. For phagocytosis of bacteria, put 100 μl cell suspension and 10 μl stationary culture Escherichia coli K12 suspension (5 x 109 bacteria/ml HBSS) in a 12 x 75 mm polypropylene test tube. Incubate at 37°C. Take 20 µl aliquots at 5, 10, 15, and 20 minutes, dilute each with 1.00 ml cold HBSS, and store on ice until staining. To stain, incubate 1.00 ml diluted cell suspension with 10 µl hydroethidine working solution for 15 minutes at 37°C.
- 5. Counterstain dead cells by incubating 250 μ l stained cell suspension with 5 μ l 3mM PI for 3 minutes on ice (final PI concentration 60 μM).
- 6. Quench dead cell fluorescence instead of counterstaining if highly active cells overlap with PI fluorescence. Add 5 µl trypan blue solution to 250 µl stained cells (final trypan blue concentration

200 µM) and run immediately.

7. Run on flow cytometer.

Excitation: 488 nm (argon laser) or high pressure mercury arc lamp with either 360-380 or 470-500 nm

bandpass filter

600 nm longpass for ethidium bromide (viable cells) and PI (dead cells) red fluorescence Filters:

optional 420-460 nm bandpass for HE blue fluorescence as a control on substrate loading

Reagents

HBSS Hydroethidine Propidium iodide Trypan blue Phorbol 12-myristate 13-acetate	Sigma Chemical Co. Molecular Probes Serva Serva Sigma Chemical Co.	# H-1387 # D-1168 # 33671 # 37252 # P-8139
---	--	--

References

Rothe, G., and G. Valet Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescin. J. Leuk. Biol. 47:440-448, 1990.

Rothe, G., and G. Valet Flow cytometric characterization of oxidative processes in neutrophils and monocytes with dihydrorhodamine 123, 2',7'-dichlorofluorescin and hydroethidine. In Advances in Analytical Cellular Pathology. G. Burger, M. Oberholzer, and G.P. Vooijs, editors. Elsevier, 1990. pp. 313-314.

Rothe, G., A. Emmendörffer, A. Oser, J. Roesler, and G. Valet Flow cytometric measurement of the respiratory burst activity of phagocytes using dihydrorhodamine 123. J. Immunol. Meth. 138:133-135, 1991.

NOTES:					
	<u></u>	No.			
			×		
		1		45	
	wa, 212,				

HANDBOOK

FLOW CD29 CD29

L. Paul Robinson, Editor

Associate Editors: Zbigniew Darzynkiewicz, Phillip Dean, Lynn Dressler, Hans Tanke, and Leon Wheeless Address all Inquiries to the Publisher Wiley-Liss, Inc., 605 Third Avenue, New York, NY 10158-0012

Copyright © 1993 Wiley-Liss, Inc.

Printed in the United States of America.

py reproduc-

Under the conditions stated below the owner of copyright for this book hereby grants permission to users to make photocop clients. tions of any part or all of its contents for personal or internal or organizational use, or for personal or internal use of specifilncorpo-This consent is given on the condition that the copier pay the stated per-copy fee through the Copyright Clearance Center, Fee List, rated, 27 Congress Street, Salem, MA 01970, as listed in the most current issue of "Permissions to Photocopy" (Publisher'snt does not distributed by CCC, Inc.), for copying beyond that permitted by sections 107 or 108 of the US Copyright Law. This consetting new extend to other kinds of copying, such as copying for general distribution, for advertising or promotional purposes, for creat collective works, or for resale.

nt and

While the authors, editors, and publisher believe that drug selection and dosage and the specification and usage of equipmeccept no devices, as set forth in this book, are in accord with current recommendations and practice at the time of publication, they & herein. In legal responsibility for any errors or omissions, and make no warranty, express or implied, with respect to material containtion relating view of ongoing research, equipment modifications, changes in governmental regulations and the constant flow of informalation to drug therapy, drug reactions, and the use of equipment and devices, the reader is urged to review and evaluate the informages in the provided in the package insert or instructions for each drug, piece of equipment, or device for, among other things, any chainstructions or indication of dosage or usage and for added warnings and precautions.

ct reproduc-

The publication of this volume was facilitated by the authors and editors, who submitted the text in a form suitable for dire tion without subsequent editing or proofreading by the publisher.

Library of Congress Cataloging-in-Publication Data

Handbook of flow cytometry methods / editor, J. Paul Robinson; associate editors, Zbigniew Darzynkiewicz ... [et al.].

p. cm.
Includes bibliographical references (p.) and index.
ISBN 0-471-59634-5

1. Flow cytometry—Laboratory manuals. I. Robinson, J. Paul.

QH585.5.F56H36 1993 574.87'028—dc20

92-47082

CIP

The text of this book is printed on acid-free paper.