CRC

IMMUNO-GOLD LABELING *in* CELL BIOLOGY

A. J. Verkleij
J. L. M. Leunissen

CRC PRESS

Immuno-Gold Labeling in Cell Biology

Editors

Prof. Dr. A. J. Verkleij
Institute of Molecular Biology
and Medical Biotechnology
State University of Utrecht
Utrecht, The Netherlands

and

Dr. J. L. M. Leunissen
Janssen Research Foundation
Janssen Pharmaceutica/Life Sciences
Beerse, Belgium

CRC Press, Inc.
Boca Raton, Florida

Library of Congress Cataloging-in-Publication Data

Immuno-gold-labeling in cell biology/editors, A. J. Verkleij, J. L. M.
Leunissen.
p. cm.
Includes bibliographies and index.
ISBN 0-8493-6053-6
1. Immuno-gold labeling. 2. Electron microscopy--Technique.
3. Immunocytochemistry--Technique. I. Verkleij, A. J.
II. Leunissen, J. L. M.
[DNLM: 1. Histocytochemistry. 2. Immunochemistry. 3. Microscopy,
Electron--methods. QW 504.5 I334]
QR187.I482I44 1989
574.8'028--dc19
DNLM/DLC 88-7436

Direct all inquiries to CRC Press, Inc., 2000 Corporate Blvd., N.W., Boca Raton, Florida, 33431.

International Standard Book Number 0-8493-6053-6

Library of Congress Card Number 88-7436
Printed in the United States

PREFACE

This book is intended to serve as a bridge between cell biologists and electron microscopists, between applications and methodology c.q. technology of immuno-gold labeling. The book is especially meant to describe and discuss recent developments of immuno-gold probe applications, preparation methods, and to demonstrate the validity but also the pitfalls of immuno-gold labeling with some important applications.

Although the preparation of immuno-gold probes may seem to have evolved into a series of standardized methods, there are still developments regarding the preparation of gold sols and the effective coupling of proteins to colloidal gold particles. This will be dealt with in the first part of the book.

The second part comprises many new and intriguing developments in electron-microscopic research using immuno-gold probes. The applicability of the probes is related to a number of preparative methods such as cryo-ultramicrotomy, freeze-substitution, and the different replica methods. In particular, pitfalls due to loss of antigenicity, penetration of the probes, steric hindrance, loss of gold particles in replicas etc., will be discussed.

Thirdly, application in cell biological research will be described and several methods and results are discussed from the point of view of cell biological interest to show the value of immuno-gold probes.

A. J. Verkleij
J. L. M. Leunissen

THE EDITORS

Arie J. Verkleij, Ph.D., is Head of the Division of Electron Microscopy and Structural Analysis and Professor of the Department of Molecular Cell Biology at the University of Utrecht, The Netherlands.

Dr. Verkleij obtained his training at the University of Utrecht, receiving his A.B. degree in 1968, his M.S. degree in 1970, and his Ph.D. degree in 1975 from the Department of Biochemistry (leader, Prof. Dr. L. L. M. van Deenen). He served as Associate Professor of Biology at the Institute of Molecular Biology and Medical Biotechnology, University of Utrecht with the following research activities: cell biology, structure and function of biological and artificial membranes, and freeze-etching techniques for electron microscopy. He completed a one year sabbatical leave as Visiting Scientist at the Biological Laboratories, Harvard University (Prof. Dr. D. Branton), where he was involved in the study of the cytoskeleton membrane interaction. After his return he continued his work on membranes, especially lipid polymorphism, membrane fusion, and ischemia, applied techniques: freeze-fracturing, cryo-ultramicrotomy, freeze-substitution, and immunolabeling. Since 1986 he has been Full Professor of Molecular Cell Biology at the Department of Molecular Cell Biology at the University of Utrecht.

Dr. Verkleij is the author of more than 200 papers and has been the author or co-author of many books. His current major research interests relate to membrane structure and dynamics, and particularly membrane receptors and ischemia.

Jan L. M. Leunissen, Ph.D., is associated with the Department of Cellular Biology and Pathology (leader Dr. M. De Brabander) of the Janssen Research Foundation at Janssen Pharmaceutica in Beerse, Belgium.

Dr. Leunissen obtained his training as a biologist at the State University of Utrecht, The Netherlands, receiving his A.B. degree in 1973, his M.S. degree in 1976, and his Ph.D. degree in 1982 in the Department of Molecular Cell Biology, division of Electron Microscopy and Structural Analysis (Prof. Dr. P. F. Elbers and Prof. Dr. H. O. Voorma). His Ph.D. study comprised the development and application of cryo-ultramicrotomy in cell biology, studying amongst other things hormone localization in the pituitary, using immunocytochemical approaches.

As part of his postdoctorate studies he was associated with this department and the Institute of Molecular Biology and was involved in localization studies of protein synthesis and transport in bacteria and of brain proteins involved in signal transduction.

Early in 1986 he joined the Janssen Research Foundation in the Department of Life Sciences/Life Sciences Products (Dr. J. R. DeMey). As an electron microscopist his current interests relate to the field of specfic localizations of drugs and the development of specific localization methods in immunocytochemistry, for example.

CONTRIBUTORS

Wolfgang Bohn, Ph.D.
Doctor
Department of Cytology and Virology
Heinrich-Pette-Institute
Hamburg, West Germany

Johannes Boonstra, Ph.D.
Doctor
Department of Molecular Cell Biology
University of Utrecht
Utrecht, The Netherlands

Frederick J. Brakenhoff, Ph.D.
Doctor
Laboratory for Electron Microscopy
University of Amsterdam
Amsterdam, The Netherlands

Ling-Yi Chang, Ph.D.
Medical Research Assistant Professor
Department of Medicine
Duke University
Durham, North Carolina

James D. Crapo, M.D.
Professor of Medicine
Department of Medicine
Duke University Medical Center
Durham, North Carolina

Guy Daneels
Department of Life Sciences
Janssen Pharmaceutica
Beerse, Belgium

Marc De Brabander, Ph.D.
Head, Division of Cellular Biology and Chemotherapy
Department of Life Sciences
Janssen Pharmaceutica
Beerse, Belgium

Jan R. De Mey, Ph.D.
Department of Life Sciences
Janssen Pharmaceutica
Beerse, Belgium

Mark De Raeymaeker
Department of Life Sciences
Janssen Pharmaceutica
Beerse, Belgium

Bart De Wever
Department of Life Sciences
Janssen Pharmaceutica
Beerse, Belgium

Steven I. Dworetzky, Ph.D.
Postdoctoral Associate
Department of Cell Biology
University of Massachusetts Medical Center
Worcester, Massachusetts

Ludwig Edelmann, Ph.D.
Department of Medical Biology
University of the Saarland
Homburg-Saar, West Germany

Carl M. Feldherr, Ph.D.
Professor
Department of Anatomy and Cell Biology
University of Florida
Gainesville, Florida

Hugo Geerts, Ph.D.
Doctor
Department of Life Sciences
Janssen Pharmaceutica
Beerse, Belgium

Staf Geuens
Department of Life Sciences
Janssen Pharmaceutica
Beerse, Belgium

Hans J. Geuze, Prof. Dr.
Department of Cell Biology
University of Utrecht Medical School
Utrecht, The Netherlands

Willem J. Hage
Hubrecht Laboratory
KNAW
Utrecht, The Netherlands

Rene Hermann, Ph.D.
Department of Cell Biology
Electron Microscopy Laboratory
Zurich, Switzerland

Heinz Hohenberg, Dipl.-Ing.,
Department of Cytology and Virology
Heinrich-Pette-Institute
University of Hamburg
Hamburg, West Germany

Marc Horisberger, Ph.D.
Head
Nestlé Research Centre
Nestec, Ltd.
Lausanne, Switzerland

Bruno M. Humbel, Ph.D.
Division of Cell Biology
Max-Planck-Institute for Biochemistry
Martinsried, West Germany

Dale W. Laird, Ph.D.
Department of Biochemistry
University of British Columbia
Vancouver, British Columbia, Canada

Gabriele Langanger, Ph.D.
Department of Cellular Biology and Chemotherapy
Laboratory of Biochemical Cytology
Janssen Pharmaceutica
Beerse, Belgium

Jan L. M. Leunissen, Ph.D.
Department of Life Sciences
Janssen Pharmaceutica
Beerse, Belgium

José Leunissen-Bijvelt
Department of Molecular Cell Biology
University of Utrecht
Utrecht, The Netherlands

Wilbers A. M. Linnemans, Ph.D.
Department of Molecular Cell Biology
State University of Utrecht
Utrecht, The Netherlands

Klaus Mannweiler, Prof.Dr.med.
Head of Department
Department of Cytology and Virology
Heinrich-Pette-Institute
Hamburg, West Germany

Marc Moeremans
Senior Researcher
Department of Life Sciences
Janssen Pharmaceutica
Beerse, Belgium

Robert S. Molday, Ph.D.
Professor
Department of Biochemistry
University of British Columbia
Vancouver, British Columbia, Canada

Arremieke M. Mommaas-Kienhuis, Ph.D.
Department of Dermatology
R. U. Leiden
Leiden, The Netherlands

Martin Muller, Dr.sc.nat.
Professor
Department of Cell Biology
Electron Microscopy Laboratory
Eidgenössische Technische Hochschule
Zürich, Switzerland

Nanne Nanninga, Ph.D.
Professor of Molecular Cytology
Department of Molecular Cell Biology
University of Amsterdam
Amsterdam, The Netherlands

Klaus Neumann, Ph.D.
Department of Medical Biology
University of the Saarland
Homburg-Saar, West Germany

Marie-Thérèse Nicolas, Dr. és Sc.
Chargée de Recherches
Laboratoire de Bioluminescence
CNRS
Paris, France

Rony Nuydens, Ph.D.
Department of Life Sciences
Janssen Pharmaceutica
Beerse, Belgium

Pedro Pinto da Silva, Ph.D.
Chief, Membrane Biology Section
Laboratory of Mathematical Biology
National Cancer Institute
Frederick, Maryland

George Posthuma, M.Sc.
Department of Cell Biology
State University of Utrecht
Utrecht, The Netherlands

Gabriel Rutter, Ph.D.
Department of Cytology and Virology
Heinrich-Pette-Institute
Hamburg, West Germany

Heinz Schwarz, Ph.D.
Max-Planck-Institute for Biology
Tübingen, West Germany

Patrick Schwarb, Ph.D.
Department of Cell Biology
Electron Microscopy Laboratory
Zurich, Switzerland

Hellmuth Sitte, Ph.D.
Professor Doctor
Department of Medical Biology
University of the Saarland
Homburg-Saar, West Germany

Jan W. Slot, Ph.D.
Centre for Electron Microscopy
University of Utrecht Medical School
Utrecht, The Netherlands

Jan Tommassen, Ph.D.
Department of Molecular Cell Biology
State University of Utrecht
Utrecht, The Netherlands

Nico van Belzen, M.Sc.
Department of Molecular Cell Biology
University of Utrecht
Utrecht, The Netherlands

Paul M. P. van Bergen en Henegouwen, Ph.D.
Department of Molecular Cell Biology
University of Utrecht
Utrecht, The Netherlands

Pim van Maurik
Hubrecht Laboratory
NIOB
Utrecht, The Netherlands

Edwin A. van Spronsen,
Department of Molecular Cytology
Vakgroep E. M.
Amsterdam, The Netherlands

Bert J. Vermeer, M.Sc.
R. U. Leiden
Leiden, The Netherlands

Arie J. Verkleij, Prof.Dr.
Institute of Molecular Biology and Medical Biotechnology
University of Utrecht
Utrecht, The Netherlands

Wim F. Voorhout, Ph.D.
Department of Cell Biology
University of Utrecht
Utrecht, The Netherlands

Paul Walther, Ph.D.
Department of Cell Biology
Electron Microscopy Laboratory
Zurich, Switzerland

Frederick A. C. Wiegant, Ph.D.
Department of Molecular Cell Biology
University of Utrecht
Utrecht, The Netherlands

TABLE OF CONTENTS

Preparation of Immuno-Gold Probes

Chapter 1

PREPARATION OF GOLD PROBES

Jan L. M. Leunissen and Jan R. De Mey

TABLE OF CONTENTS

I. INTRODUCTION

Among (immuno)cytochemical marker systems, colloidal gold particles have undergone an enormous evolution since their introduction by Faulk and Taylor.[15] This evolution has been especially encouraged by the applicability of colloidal gold probes in electron microscopic investigations. Several reviews cover the aspects of sol preparation, probe preparation, and procedures for the use of gold probes in cytochemical studies.[3,11,12,22,31,35] The present chapter aims to summarize the most important aspects of gold sol preparation, probe preparation, and application. A survey of an alternative method based on semicovalent binding of macromolecules to dextran derivative-coated gold particles can be found in Chapter 3.

Colloidal gold particles are well suited as a marker system for the following reasons:

1. Colloidal gold particles can be easily prepared in a wide range of sizes, from 2 to 3 nm to well above 100 nm.
2. The latest published methods for gold sol preparation provide narrow size distributions in the range between 3 and 15 nm. If the size distribution (coefficient of variance) is below 15%, the sol is considered as monodisperse. Monodisperse sols are suited for double labeling localization studies with the least possible overlap in sizes, even with diameters down to approximately 3 nm.
3. Gold probes, prepared by coupling of macromolecules (mostly proteins) to colloidal gold particles, generally retain the specific activities of the macromolecules. The probes may be stored over longer periods of time, or even frozen in the presence of glycerol or sucrose.
4. Gold particles are very distinctly visualized as dense structures within biological entities in the transmission electron microscope. A particulate labeling allows the immunoreactivity to be interpreted in ultrastructural terms.
5. Gold particles can be excited to emit secondary electrons or to "back-scatter" electrons, making them useful markers in scanning electron microscopy.[8,21]
6. Colloidal gold particles are not only intensely colored, they can also reflect light or may be used as nuclei for the deposition of silver, thus establishing their usefulness in light microscopy as well as in immunoblotting.[7,9,10,27] The intrinsic contrast of colloidal gold particles allows their visualization as particulate entities in special microscopic techniques (see Chapters 12 and 13).
7. Gold probes are preferred to radioactively and enzymatically labeled probes because of the improved resolution and because gold probe activity is only dependent on retention of the specific probe protein activity. In addition, they are nonhazardous reagents.

The latter emphasizes the broad usefulness of colloidal gold particles as a uniform marker system for a wide range of immunochemical detection methods. This makes it possible to correlate results obtained through various approaches: light microscopy, transmission electron microscopy, scanning electron microscopy, and immunoblotting.

II. PREPARATION OF COLLOIDAL GOLD PARTICLES

Colloidal gold particles are formed by chemical reduction of an aqueous solution of tetrachloroauric acid (condensation method). The following events characterize the reduction process and determine the ensuing characteristics of the colloid particles: the nucleation, the crystal growth, and the reaction speed. Upon onset of the reduction process, gold atoms are liberated from tetrachloroauric acid. These gold atoms tend to aggregate in order to minimize the free surface energy (crystallization process), thus forming microcrystals. During pro-

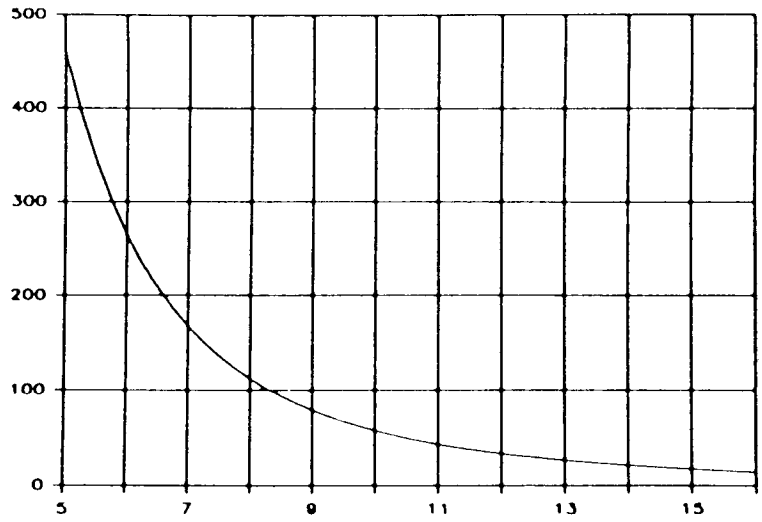

FIGURE 1. Relationship between the average number of gold particles per milliliter gold sol prepared from a 0.01% solution of tetrachloroauric acid and the average diameter of the gold sol particles (in the range of 5 to 15 nm). X-axis, particle diameter (nm); Y-axis, number of particles, 10^{11}/ml.

gressing reduction, the microcrystals grow further until all tetrachloroauric acid has been reduced[16] and colloid formation is completed. The type of reducing agent used and the concentration of the reacting components largely determine the ratio between nucleation and growth, and thus the final particle size and size distribution. Reductors that are about the most widely used are white phosphorous[14,43] and sodium citrate.[16,43] Next to these, sodium borohydride[40] and thiocyanate[2] can be used as reductors for the preparation of colloidal gold particles smaller than 5 nm with a lower limit of approximately 2 nm. Although the detectability of such particles in the electron microscope may be limited due to reduced electron density, they may nevertheless provide a basis for optimal penetration. Colloidal gold particles can be increased in size by condensation of gold on the particle surface.[6,44] Recently, a method has been published for the controlled growth of existing gold particles using reduction by the white phosphorous procedure.[6] The particle diameter, initially 5.5 nm, can be gradually increased to approximately 12 nm by repetitive reduction cycles. In this way, sols can be prepared with extremely high particle density and a low diameter spreading. The particles prepared through the controlled growth procedure showed improved reactivity in immunocytochemical studies when compared to ascorbate-reduced particles.[38] This idea of controlled growth has also been elaborated by Slot and Geuze[36] as a modification of the method published by Muhlpfordt[29] using tannic acid and sodium citrate. By using a mixture of a fast reductor (tannic acid) and a slow reductor (citrate), more or less a separation in nucleation and growth is achieved. By varying the amount of tannic acid, there is a corresponding variation in amount of nuclei and thus in diameter of the resulting colloidal particles, provided the amount of tetrachloroauric acid is constant. A reason for the narrow size distribution may be found in the fact that tannic acid partly stabilizes the colloid. Reduction under stabilizing conditions is a well-known method to limit diameter spreadings.

According to Frens,[16] all the tetrachloroauric acid is converted to metallic gold, offering the possibility to calculate the amount of gold particles per unit volume, thus providing a basis for quantification. Figure 1 shows the relation between the number of gold particles per unit volume and the mean particle diameter, provided the same amount of tetrachloroauric acid is reduced.

Since the maximum light adsorption (at 520 nm) is almost independent of the particle size (see also Reference 6), this implies that for sols and probes of the same OD520 nm (the optical density of the sols prepared according to the standard recipes is approximately 1), the number of particles decreases by a factor of 8 and the total particle surface decreases by a factor of 2 for each doubling in size. This has important implications for the stabilization of the sol (i.e., the minimum amount of macromolecules necessary to prevent electrolyte-induced flocculation), and for the efficiency of the probe in dependency of the particle size.

A. Preparation of Colloidal Gold Particles: Recipes

General remark — The success of reproducible gold sol preparation is strongly dependent on the use of meticulously cleaned glassware and ultrapure water, both for the preparation of the solutions and for the last rinsing of the glassware. The use of freshly prepared, twice deionized, glass distilled water is recommended.

1. Reduction by White Phosphorus

Reduction by white phosphorus, after Faulk and Taylor[15] as modified by Slot and Geuze;[34] mean particle size: 5 nm.

1.5 ml of a 1% $HAuCl_4$ solution and 1.4 ml of a 0.1 *M* K_2CO_3 solution are added to 120 ml of distilled water. To this solution, 1 ml of a phosphorus/ether solution is added. The pipette tip is submerged to provide for minimal air contact of the white phosphorus. The phosphorus solution is prepared by adding one part of ether saturated with white phosphorus to four parts of ether. The mixture is left for 15 min at room temperature and boiled under reflux until the color changes from brownish to red.

2. Reduction by Sodium Citrate — Method 1

Reduction by sodium citrate, after Frens;[16] particle sizes: 16 to 150 nm.

50 ml of a 0.01% $HAuCl_4$ solution is heated to boiling and 0.15 to 1 ml of a 1% sodium citrate solution is added and boiling is continued. In about 25 s, the nucleation starts and the solution turns faintly blue. After 70 s to 15 min, which is inversely related to the amount of the added citrate solution, the solution turns red and sol formation is complete. The diameters can be derived from the following table:

Amount of citrate (ml)	Diameter (nm)	Sol color
1.00	16	Orange
0.75	24.5	Red
0.50	41	Red
0.30	71.5	Dark red
0.21	97.5	Violet
0.16	147	Violet

Reproducibility is the highest with the lower particle diameters.

3. Repetitive Reduction by White Phosphorus

Repetitive reduction by white phosphorus, after van Bergen en Henegouwen and Leunissen;[6] particle size: 5 to 12 nm.

Starter sol (5.6 nm), 1.5 ml of a 1% $HAuCl_4$ solution, 1.2 ml of a 0.1 *M* K_2CO_3 solution, in 120 ml distilled water.

The mixture is stirred for at least 3 min until the yellowish color has disappeared. While stirring vigorously, 1.0 ml of a freshly prepared 20% saturated solution of white phosphorus in diethylether is added. Within 5 min, the mixture's color changes to brown/red. The suspension is boiled under reflux until the color has changed to orange/red (within 10 min).

The white phosphorus/ether solution is prepared from a 100% stock solution. To prepare the stock solution, a few pieces of white phosphorus are added to 20 ml of diethylether and stirred for 1 h at room temperature. The solution is cleared by adding distilled water and mixing. The stock is stored at −20°C.

Growth of starter sol particles — An aliquot of 0.5 ml of a freshly prepared 20% saturated white phosphorus/ether solution is added to 60 ml of the starter sol at 4°C with vigorous stirring. After 5 min, 0.75 ml of a 1% $HAuCl_4$ solution and 0.6 ml of a 0.1 *M* K_2CO_3 solution are added. The color of the suspension turns brownish/red. Subsequently, the sol is boiled under reflux for 10 min, by which time the sol color has turned red. The diameter can be further increased by repeating the procedure as described or by adding larger amounts of the above mentioned solutions in stoichiometric quantities. An indication of the obtainable average diameters and standard deviations can be obtained from the table below.

Average diameter (nm)	Standard deviation	Number of additional reductions
5.6	0.9	0
6.7	1.0	1
7.9	0.9	2
9.8	1.3	4
12.1	1.0	6

4. Reduction by Tannic Acid and Sodium Citrate

Reduction by tannic acid and sodium citrate, after Slot and Geuze;[36] particle sizes: 3 to 17 nm. 100 ml gold sol is made up of two solutions: (1) 1 ml of a 1% $HAuCl_4$-solution in 79 ml distilled water; and (2) 4 ml of a 1% tri-sodium citrate·$2H_2O$ solution, 0 to 5 ml of a 1% tannic acid solution, 0 to 5 ml of a 25 m*M* K_2CO_3 solution (same amount as the 1% tannic acid solution) distilled water to bring the volume to 20 ml.

Both solutions are brought to 60°C. Then solution 2 is quickly added to solution 1 while stirring. The mixture is kept at 60°C until the colloid formation is completed. Sol formation occurs within 1 s when high amounts of tannic acid are added, but the reaction time increases with decreasing amounts of tannic acid. After completion of sol formation the sols are heated until boiling.

Remark: the source of tannic acid is important as the chemical composition varies among brands. The tannic acid used in this recipe is obtained from Mallinckrodt, St. Louis, MO, code 8835.

5. Reduction by Sodium Citrate — Method 2

Reduction by sodium citrate, after Moeremans et al.,[45] average particle diameter: 8 to 10 nm.

Pour 232.50 ml of distilled deionized water in a 500 ml erlenmeyer flask. Heat to boiling and start refluxing. Add 15 ml of a 1% sodium citrate solution. Reflux for 5 min. Subsequently add 2.5 ml of a 1% $HAuCl_4$ solution rapidly and under vigorous stirring. Reflux for another 15 min.

III. RATIONALE OF GOLD PROBE PREPARATION

Colloidal gold particles carry a net negative surface charge. This property is exploited in the low pH staining of positively charged electrophoretically separated protein bands on cellulose-based membranes after electroblotting.[28]

Positively charged proteins in solution will also tend to form complexes after mixing with colloidal gold particles by electrostatic interactions. However, since these complexes are largely aggregates, they are not, or are at the least, less suitable as (immuno)gold probes