AD NUMBER

ADB281773

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Jul 2001. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St., Ft. Detrick, MD 21702-5012.

AUTHORITY

USAMRMC ltr, 8 Jan 2003

AD_____

Award Number: DAMD17-98-1-8182

TITLE: A Novel Technique to Follow Consequences of Exogenous Factors, Including Therapeutic Drugs, on Living Human Breast Epithelial Cells

PRINCIPAL INVESTIGATOR: Carolyn A. Larabell, Ph.D.

CONTRACTING ORGANIZATION: University of California at Berkeley
Berkeley, California 94720

REPORT DATE: July 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-98-1-8182
Organization: University of California at Berkeley

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

7/15/02

_____ _____

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Final (1 Jul 98 - 30 Jun 01)

4. TITLE AND SUBTITLE
A Novel Technique to Follow Consequences of Exogenous Factors, Including Therapeutic Drugs, on Living Human Breast Epithelial Cells

5. FUNDING NUMBERS
DAMD17-98-1-8182

6. AUTHOR(S)
Carolyn A. Larabell, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California at Berkeley
Berkeley, California 94720

E-mail: larabell@lbl.gov and pmgale@lbl.gov

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Jul 01). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The goal of this research was to develop a technique for imaging living human breast epithelial cells in 3-D cultures and to evaluate their responses to the application of exogenous factors. Most model systems used to study breast cancer utilize cells growing in monolayers on plastic substrates. Although a great deal of information about cells and their responses to exogenous agents, such as therapeutic drugs, can be learned from these studies, there are also major limitations to this approach. In short, cells growing on plastic are flat, whereas cells in the body are three-dimensional. Recent data from a number of laboratories demonstrate that cells growing in monolayers do not necessarily respond to exogenous substances in the same manner as cells growing in 3-D (Weaver et al., 1997). Therefore, we developed technology for imaging human mammary epithelial cells growing in a three-dimensional reconstituted basement membrane. This technique enables monitoring fluorescently labeled proteins in living "normal," premalignant and tumor cells. This approach can be used to detect rapid, "real-time" responses by these cells to the effects of a spectrum of exogenous factors, including therapeutic agents.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Living human mammary epithelial cells, confocal microscopy, assay	13
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

The goal of this research was to develop a technique for imaging living human breast epithelial cells in 3-D cultures and to evaluate their responses to the application of exogenous factors. Most model systems used to study breast cancer utilize cells growing in monolayers on plastic substrates. Although a great deal of information about cells and their responses to exogenous agents, such as therapeutic drugs, can be learned from these studies, there are also major limitations to this approach. In short, cells growing on plastic are flat, whereas cells in the body are three-dimensional. Recent data from a number of laboratories demonstrate that cells growing in monolayers do not necessarily respond to exogenous substances in the same manner as cells growing in 3-D (Weaver et al., 1997). Therefore, we developed technology for imaging human mammary epithelial cells growing in a three-dimensional reconstituted basement membrane. This technique enables monitoring fluorescently labeled proteins in living "normal," premalignant and tumor cells. This approach can be used to detect rapid, "real-time" responses by these cells to the effects of a spectrum of exogenous factors, including therapeutic agents.

BODY

Specific Aim 1: Visualization of Living Human Mammary Epithelial Cells Growing in a Three-Dimensional Matrix

Tracking proteins in live human mammary epithelial cells growing in three-dimensional reconstituted basement membrane components (i.e. Matrigel) is more complex than tracking them in two-dimensional cultures. As we discussed in our previous report, the use of confocal microscopy solved some of the optical problems involved in this process, making it feasible to examine specific organelles labeled with vital dyes in living cells. However, tracking proteins tagged with the green fluorescent protein (GFP) introduced a number of additional problems. Examining fluorescent proteins in living cells can be problematic whether the cells are growing in 2-D or 3-D cultures. The lasers utilized to excite the fluorophore can themselves damage the cells and excitation of the fluorophore can also generate photoxidants that are toxic to the cells. Extreme caution must be used for these studies to assure that one is studying a healthy cell. The best approach currently available for examining fluorescent proteins in thick (100 to 200 micron) cultures is multi-photon microscopy. We purchased the Zeiss 510 NLO system, which was installed in October 2000. This solved many of the problems we had encountered (One still must be cautious! New problems associated with IR excitation, such as excessive heat, can occur.) We were then able to examine the protein beta-catenin, as outlined in the proposal, in living cells.

Tracking the GFP protein construct of beta-catenin also turned out to be problematic. As discussed in our previous report, cells examined at early time points after transfection with beta-catenin-GFP demonstrated the presence of small foci of beta-catenin in the nucleus. At later time points, large clusters of beta-catenin-GFP were seen

in the nucleus of the cells, but the cells had become rounded and demonstrated several projections from the cell surface that extended and retracted at multiple sites around the cell. Additional studies since have demonstrated that all cells transfected with beta-catenin-GFP undergo apoptosis. This has been confirmed both biochemically and using imaging techniques (See Figures 1 and 2). These results are specific to transfection with the protein beta-catenin, since transfection with either the empty GFP vector or actin-GFP failed to induce apoptosis. An abstract reporting these results has been submitted to a Keystone symposium on Wnt and Beta-Catenin Signaling in Development and Disease to be held in March 2002, and a manuscript is in preparation.

Specific Aim #2: Examination of the Effects of Exogenous Factors on Living Human Mammary Epithelial Cells Growing in a Three-Dimensional Matrix.

Metastasis is a major problem associated with cancer and is related to the ability of cells to migrate. By examining live cells that had been transfected with actin-GFP (normal and tumor cells) using time-lapse imaging, the increased motility and migratory behaviors of the tumor cells became quite evident. Unfortunately, the ability to document and quantify these behaviors in living cells is limited. Existing techniques involve time-lapse videos of a limited number of cells (Rajah et al., 1998), which makes it difficult to obtain statistically significant studies of cell populations. Improved statistics can be obtained with the "scratched wound method" (Bürk, 1981; Környei et al, 2000), in which a region of the cell culture substrate is denuded of cells and then the time scale for the filling of this "hole" is observed. Unfortunately, the history of the cell migration paths is lost, and the analysis is complicated by subjective analysis of the complex and variable patterns of cell motion that lead to hole filling. A significant advance occurred with the development of the Boyden Chamber invasion assay, in which cells are seeded on one side of a membrane, and the number of cells reaching the other side is determined (Boyden, 1962; Yao et al., 1990). This method, though widely used, is extremely laborious and requires that the cells be fixed, and thus destroyed, preventing real time variation of the external conditions.

In collaboration with Prof. Paul Alivisatos, a chemist at the University of California at Berkeley, we developed a technique to use colloidal quantum dots to monitor cell motility. These quantum dots are protein-sized crystals of inorganic semiconductors that are robust and efficient light emitters (Bawendi, et al., 1990; Alivisatos, 1996). When coated with a suitable solubilizing layer, such as silica, they are stable under physiological buffer conditions (Gerion et al., 2001). We showed that the colloidal semiconductor nanocrystals are ingested by a wide variety of cells, while remaining fully luminescent, thus enabling the tracking of dynamical phenomena inside cells over periods of weeks. In addition, we demonstrated that this property can be used for quantum dot based imaging of phagokinetic tracks (see Appendix for manuscript). Additional experiments not included in our submitted manuscript are in progress to demonstrate the ability to use the quantum dots as an assay for examining cell motility and invasiveness in living cells growing in 3-D cultures. The use of the quantum dots also provides an excellent assay for the effects of exogenous factors on living human

mammary epithelial cells growing in both two and three-dimensional cultures. (A patent on this technique has been filed; see attached Report of Inventions and Subcontracts).

In another set of experiments, we used this assay to demonstrate the ability of a peptide obtained from a plant to block cell motility (manuscript in preparation; see Figure 3). The peptide, referred to as SPIKE, bundles actin. It was isolated from a plant protein by Drs. Steven Huber and Heike Winter, collaborators from North Carolina State University. In another set of experiments in which we injected the peptide into *Xenopus laevis* eggs shortly after fertilization, we demonstrated the ability of this peptide to block cell division as well. Embryos injected with the control, inactive peptide cleaved normally and were fixed at the 8-cell stage. Those embryos injected with SPIKE failed to complete cleavage. Cleavage furrows were formed, but appear to become "fixed," preventing complete cell division and blastomere formation. Labeling with rhodamine phalloidin, which binds filamentous actin, demonstrates the presence of increased amounts of actin in the abortive cleavage furrows.

KEY RESEARCH ACCOMLISHMENTS:

- Demonstrated the ability to track protein constructs tagged with green fluorescent protein (GFP) in three-dimensional matrix (Matrigel)
- Demonstrated that over-expression of beta-catenin in human mammary epithelial tumor cells (MCF-7) induces apoptosis.
- Developed fluorescence-based assay using colloidal quantum dots to measure cell motility and migration in living cells growing in two- and three-dimensional cultures.
- Used the quantum dot assay to demonstrate the ability of a peptide derived from a plant protein to block cell motility and cytokinesis.

REPORTABLE OUTCOMES

- **Manuscripts**
  - Parak, W.J., Boudreau, R., Le Gros, M.A., Gerion, D., Zanchet, D., Micheel, C.M., Williams, S.C., Alivisatos, A.P. and Larabell, C.A. (2001) Cell Motility and Metastatic Potential Studies Based on Quantum Dot Imaging of Phagokinetic Tracks. *Advanced Materials*. Submitted.
  - Boudreau, R., Engel, B., Le Gros, M.A., and Larabell, C.A. Overexpression of ß-catenin Promotes Apoptosis in Human Mammary Epithelial Tumor Cells. Manuscript In Preparation.
  - Winter, H., Boudreau, R., Holtgraewe, D., Huber, S., and Larabell, C.A. Reorganisation of actin cytoskeleton by SPIKE blocks cell motility and inhibits cytokinesis. Manuscript in Preparation.

- **Presentations**
  - "Tracking GFP-tagged proteins in human mammary epithelial cells in culture." Poster, Era of Hope, June 2000.
  - "Measuring cell motility and metastatic potential with quantum dots." Gordon Conference, *Lasers in Medicine and Biology.* July 2002.

- **Patents**
  - "Cellular Imaging Using Semiconductor Nanocrystals," IB-1755P. Carolyn A. Larabell, Mark Le Gros, Rosanne Boudreau, Wolfgang J. Parak, A. Paul Alivisatos.

- **Invention Disclosure**
  - "A synthetic peptide that causes bundling of filamentous actin (F-actin) in vitro and in situ," JIB-1571. Steven C. Huber, Heike Inge Ada Winter, Carolyn A. Larabell

CONCLUSIONS

Imaging living cells reveals data that could not readily be obtained using other techniques. We are developing techniques for imaging human mammary epithelial cells growing in 3-D cultures and for tracking GFP protein constructs in these cells. We expect this approach will yield valuable information about the responses of tumor cells to exogenous agents that would not otherwise be generated from studying fixed specimens. The use of new imaging techniques, specifically the multi-photon microscope, has enabled us to conduct experiments on cells growing in thick (up to 200 microns), three-dimensional matrices. However, our attempt to study the protein beta-catenin in normal and tumor cells by introducing a GFP construct of this protein into cells led to an entirely unexpected outcome. Over-expression of beta-catenin induced apoptosis in the cells. This, in itself, is an interesting and reportable outcome of the experiments. But it also demonstrates the hazards of studying exogenous proteins, especially those with signaling capacity; over-expression of the protein one is attempting to study may actually alter the behavior of the cells.

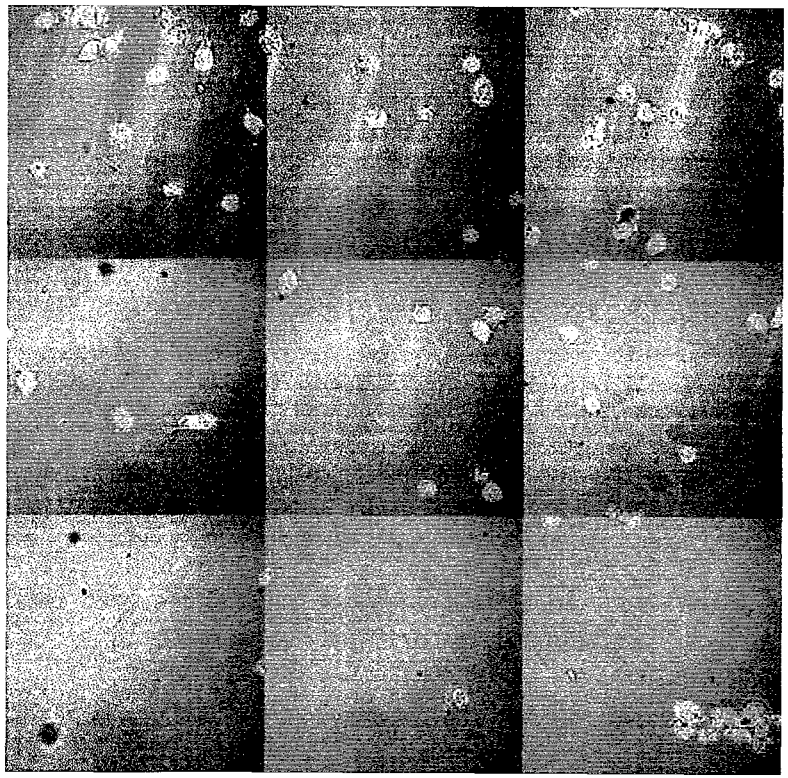
We were very successful in developing an assay for monitoring the effects of exogenous factors on living human mammary epithelial cells growing in two- and three-dimensional cultures. In collaboration with Prof. Paul Alivisatos, a chemist at the University of California at Berkeley, we developed a fluorescence-based assay that uses colloidal quantum dots to monitor cell motility and migration and, when applied to three-dimensional extracellular matrices, can be used to measure invasiveness. The strengths of this assay are: 1) It can be used with living cells; 2) It allows monitoring changes in cell behaviors in response to addition of exogenous agents; 3) There is no processing required, eliminating loss of cells and making it more accurate; and 4) It can be quantitative. We anticipate this will be an extremely useful assay for cell biologists studying cell motility as well as for cancer biologists.

REFERENCES

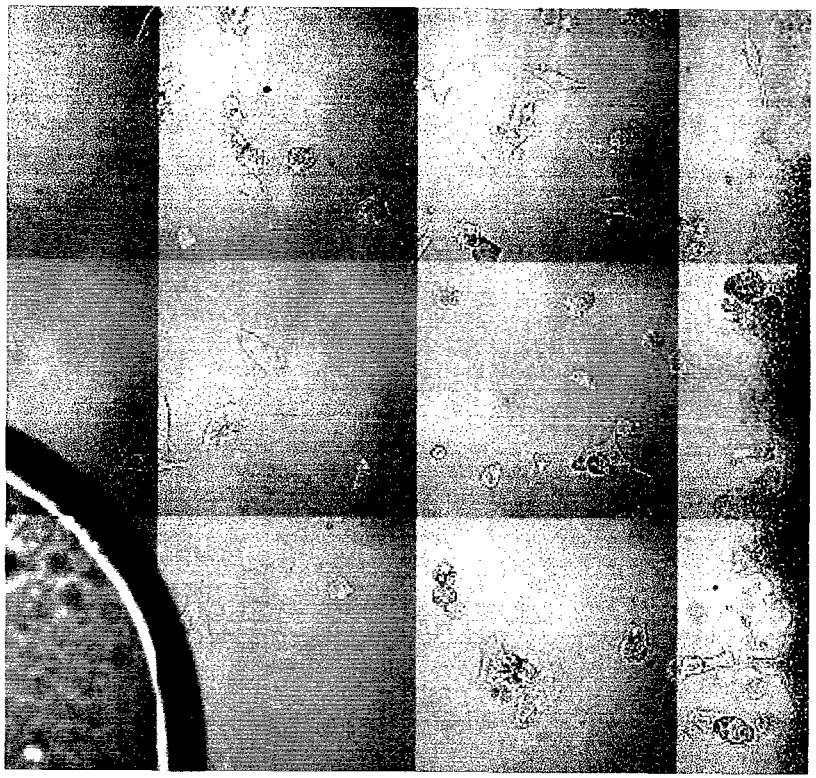
1) Alivisatos, A.P. *Science* **271**, 933-937 (1996).
2) Bawendi, M.G., Steigerwald, M.L., Brus, L.E. *Ann. Rev. Phys. Chem.* **41**, 477-496 (1990).
3) Boyden, S. *J. Exp. Med.* **115**, 453-466 (1962).
4) Bürk, R.R. *PNAS* **70**, 369-372 (1981).
5) Gerion, D., Pinaud, F., Williams, S.C., Parak, W.J., Zanchet, D., Weiss, S., Alivisatos, A.P. *J. Phys. Chem. B* **105** (37), 8861-8871 (2001).
6) Környei, Z., Czirok, A., Vicsek, T., Madarasz, E. *J. Neurosci. Res.* **61**, 421-429 (2000).
7) Rajah, T.T., Abidi, S.M.A., Rambo, D.J., Dmytryk, J.J., Pento J.T. *In vitro cell. Dev. Boil. – Animal.* **34**, 626-628 (1998).
8) Yao, J., Harvath, L., Gilbert, D.L., Colton, C.A. *J. Neurosci. Res.* **27**, 36-42 (1990).

Figure 1

MCF 7 Cells Transfected With Beta-Catenin-GFP Undergo Apoptosis



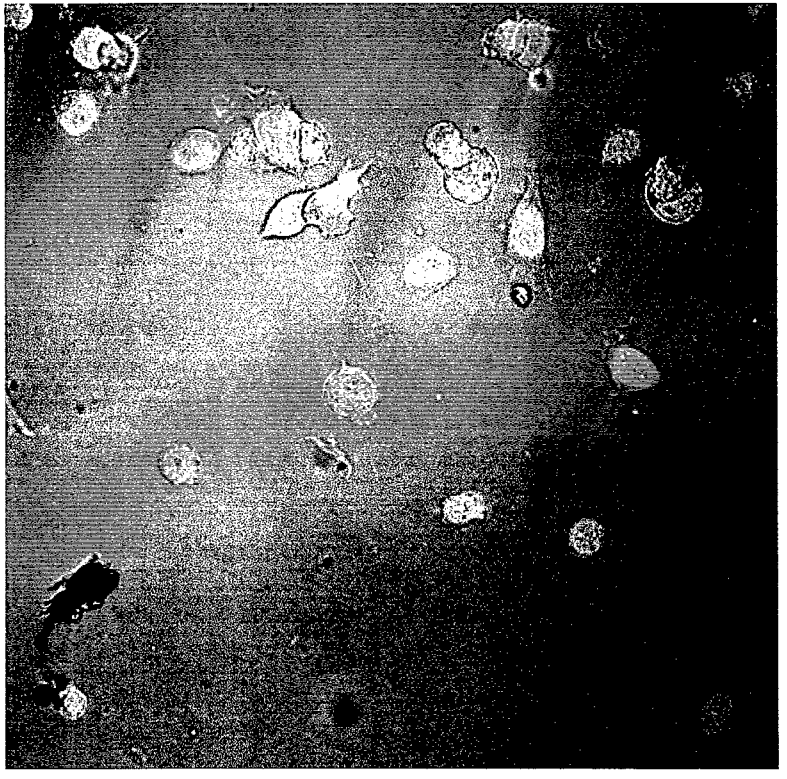
Transfected
Apo-tag positive



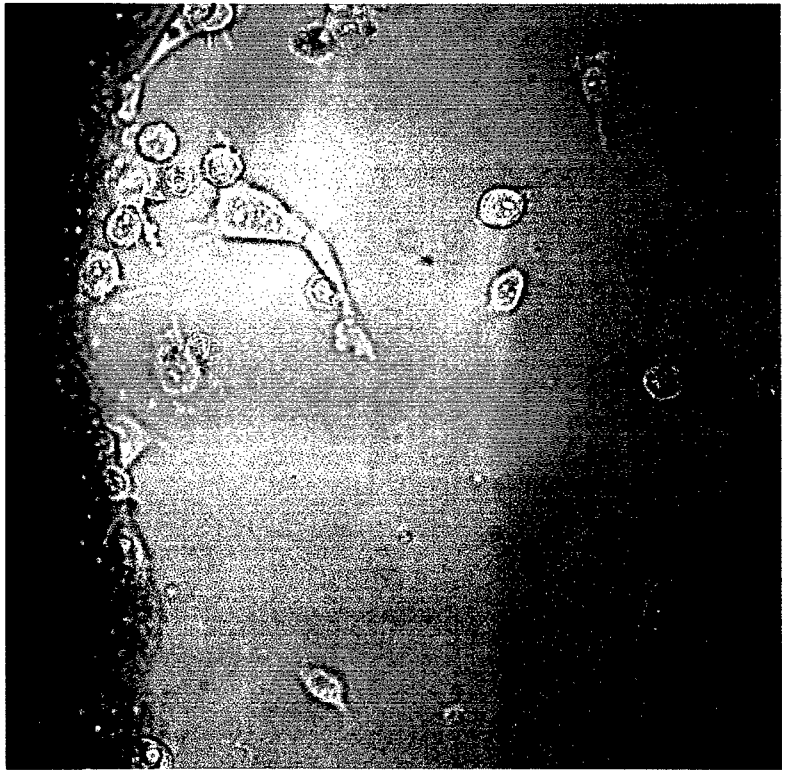
Not transfected
Apo-tag negative

Figure 2

MCF 7 Cells Transfected With Beta-Catenin-GFP Undergo Apoptosis



Transfected
Apo-tag positive



Transfected
Control (NSB)

Figure 3

Effect of Synthetic Peptide on Cleavage of Xenopus Embryos

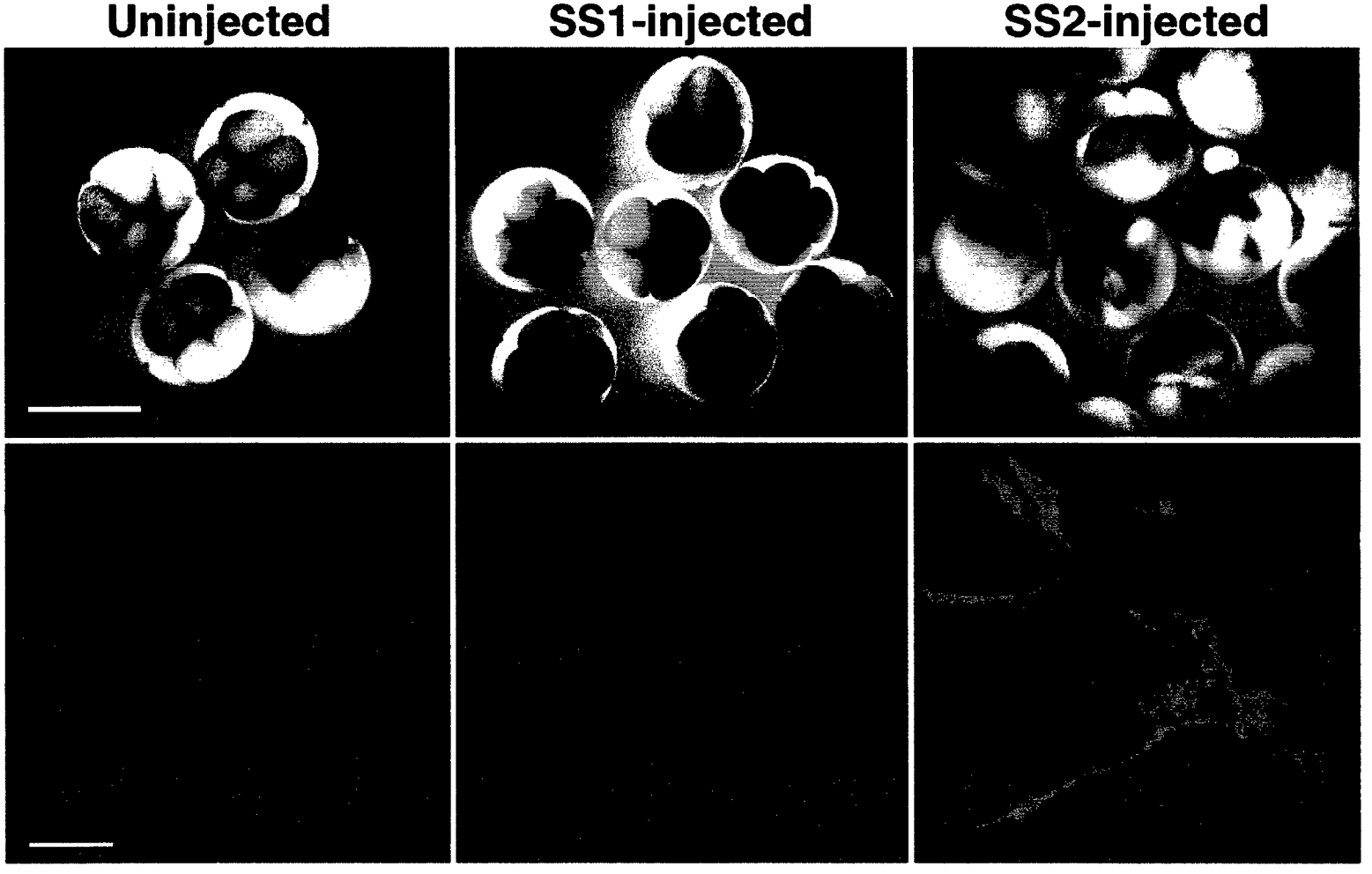
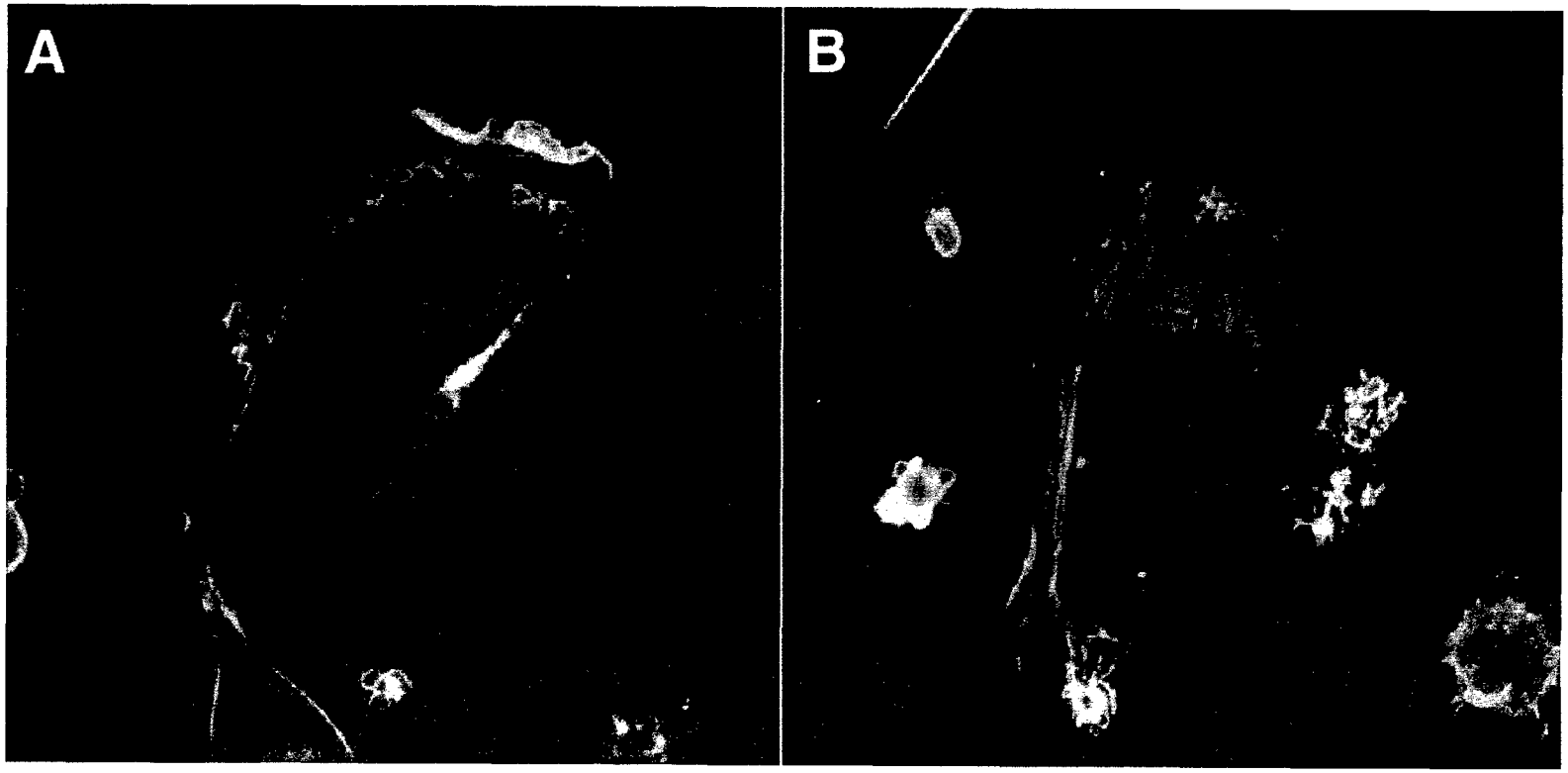


Figure 4

Treatment of Tumor Cells with Plant Compound that Stops Cell Crawling



Untreated

Treated

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 8 Jan 2003

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the enclosed. Request the limited distribution statement for the enclosed be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

ADB265840	ADB266633	ADB282069
ADB279138	ADB251763	ADB265386
ADB264578	ADB281601	ADB282057
ADB281679	ADB258874	ADB258251
ADB281645	ADB281773	ADB264541
ADB261128	ADB281660	ADB241630
ADB261339	ADB259064	ADB281924
ADB273096	ADB266141	ADB281663
ADB281681	ADB281664	ADB281659
ADB259637	ADB258830	
ADB256645	ADB266029	
ADB262441	ADB281668	
ADB281674	ADB259834	
ADB281771	ADB266075	
ADB281612	ADB281661	