purified HIS6-calmodulin fusion protein were incubated with 1 U of enterokinase (Stratagene) at room temperature for 0, 2, 4 and 10 h, respectively. A fraction of the sample was electrophoresed on a 4%–20% Tris-glycine-acrylamide gel (Novex, San Diego, CA, USA) and stained with Coomassie Blue. Figure 2A shows the expression and purification of 6x(His)-calmodulin fusion protein. The level of induced expression of 6x(His)-calmodulin is estimated to be 5%–10% of the total soluble protein. Note the extra bands appearing on the gel are probably due to the endogenous His proteins contained within the cell that are co-purified with the target. As shown in Figure 2B, calmodulin protein without any extraneously added amino acid residues was obtained by removing the 6x(His) purification tag with enterokinase. Before enterokinase cleavage, the fusion protein was dialyzed against the enterokinase cleavage buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM CaCl2) using Amicon® Microcon® Microconcentrators (Millipore, Bedford, MA, USA). The FLAG epitope can be targeted to the protein of interest and used in immunoprecipitation experiments. The 6x(His)-FLAG-calmodulin fusion protein was detected through a western blot using the M2 anti-FLAG antibody (Scientific Imaging [Eastman Kodak]) (data not shown).

In conclusion, the new pESP-5 S. pombe expression vector allows efficient and directional cloning of PCR products immediately downstream of the enterokinase recognition/cleavage site. Thus, polypeptides without extraneously added amino acid residues can be obtained by removing the purification tag with enterokinase after purification of the recombinant 6x(His) fusion protein.

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Black Cellular Spreading and Motility Assay
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Here, we describe a new technique to study cell spreading and motility on black ink particle-coated substrates. The experimental technique is simple and is based on the observation that cells migrating on a glass substrate, which is densely and evenly coated...
with ink particles, clear the particles out of their way. In this fashion, the cells produce particle-free tracks. We have entitled this assay the black cellular spreading and motility (BCSM) assay. Using this BCSM assay, we demonstrate that epidermal growth factor (EGF) induces cell spreading and motility of the human lung cancer cell lines H322 and A549. In contrast, a ligand-blocking monoclonal antibody (MAb) against the EGF-receptor (EGF-R) inhibits cell spreading and motility of these cell lines.

The H322 and A549 cells were obtained from ATCC (Rockville, MD, USA). Cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and incubated at 37°C in a 5% CO₂ atmosphere. H322 cells display an epithelial-like morphology, whereas A549 cells display an epithelial morphology. Both cell types form multilayered islands of cells.

Five milliliters of Drawing Black Ink (Staedtler; Marsmatic R, Nurnberg, Germany)/phosphate-buffered saline (PBS) buffer (1:150) solution were plated in 60-mm dishes (Nalge Nunc International, Rochester, NY, USA) for 1 min. The solution was then aspirated from the dishes. The dishes were air-dried for 2 h and sterilized in a Model 1800 Stratalinker® UV Crosslinker (Stratagene, La Jolla, CA, USA) for 10 min. Approximately 4 x 10⁴ cells were plated in 5 mL DMEM 5% FBS in the ink-coated, 60-mm tissue culture dishes. One day later, the medium was changed either with fresh medium (control), medium with 1 µg/mL of Anti-EGF-R MAb (LA1; mouse monoclonal IgG1, reacts with the external domain of EGF-R; Upstate Biotechnology, Lake Placid, NY, USA) or 10 ng/mL of EGF. One microgram per milliliter of mouse Anti-IgG1 (Becton Dickinson Canada, Mississauga, ONT, Canada) was used as a control for the effects of LA1 MAb. Morphological changes were examined by phase-contrast microscopy. The cells were examined every day for 3 days. Photographs of cells were taken from a microscope with a 10x objective and scanned using an Arcus® II scanner (Agfa, Ridgefield, NJ, USA). The images were then saved as tagged image format files (TIFF) using the Agfa Photolook® 3.0 software (Agfa), which were, in turn, used to quantify particle-free tracks produced by the cells using the NIH Image analysis program (developed at the Research Services Branch of the National Institutes of Health) called Scion Image for Windows®, PC version (http://rsb.info.nih.gov/nih-image/download.html).

Recently, we demonstrated that treatment for 3 days with 1 µg/mL of LA1 decreases cell proliferation, upregulates expression of the cell-cell adhesion molecule E-cadherin and leads to morphological changes in H322 cells (3). In contrast, EGF induces an epithelial-fibroblastoid conversion and reduces the expression of E-cadherin (Reference 3 and Al Moustafa, Urbani and O’Connor-McCourt, unpublished data). To determine whether the changes in morphology and E-cadherin levels that are induced by activation or blocking of the EGF-R affects cell spreading and motility of the H322 and

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**Figure 1. Analysis of EGF-R modulation on cell motility by the BCSM assay.** Treatment for 2 days with 10 ng/mL of EGF-induced cell spreading and motility of the H322 cell line. On average, each cell cleared ink particles (small arrows) over an area of 39.2 µm²/24 h (activation of EGF-R). In contrast, the anti-EGF-R MAb LA1 inhibited cell spreading and motility in these cells after 2 days of treatment. On average, these cells cleared ink particles over an area of only 1.2 µm²/24 h (blocking of EGF-R).
A549 cell lines, we used the BCSM assay. Figure 1 shows that treatment for 2 days with 10 ng/mL of EGF induces cell spreading and motility of the H322 cell line, whereas the anti-EGF-R MAb inhibits cell spreading and motility. The quantification of cell motility revealed that cells cleared ink particles over an area of 39.2 µm²/24 h on average per cell when treated with EGF; untreated cells cleared 29.4 µm²/24 h (data not shown). In contrast, cells treated with anti-EGF-R MAb LA1 only cleared 1.2 µm²/24 h on average per cell, i.e., LA1 reduced the cell motility of H322 cells by 96.03% compared to untreated cells and 97.02% compared to cells treated with EGF. In addition, we show that different cell lines produced different particle-free tracks, i.e., different patterns of cell spreading and motility in culture using the BCSM assay. It is clear from Figure 2 that A549 cells had a higher motility than H322 cells and that the tracks were longer and narrower. The quantification of cell auto-motility revealed that cells cleared ink particles over an area of 200.7 µm²/24 h on average per cell.

Here, we present evidence demonstrating that EGF induces the spreading and motility of the human lung cancer cell lines H322 and A549. Similarly, in colon carcinoma cells, Solic and Davies (5) have reported that EGF reduces E-cadherin expression and induces motility. Recently, Qin and Kurpakus (4) showed that transforming growth factor-alpha (TGF-α) or EGF stimulate cell motility of primary corneal epithelial cells. We now demonstrate for the first time, to our knowledge, using the BCSM assay that the opposite also holds true, e.g., that anti-EGF-R MAb inhibits cell spreading and motility of the H322 and A549 cell lines (Figure 1 and Al Moustafa, Urbani and O’Connor-McCourt, unpublished data).

In 1977, Albrecht-Buehler (1) described a technique of visualizing tracks of cultured cells moving on glass substrate treated with bovine serum albumin (BSA) and covered with gold particles. This author suggested that the phenomenon of track formation on gold particle-coated substrates be called phagokinetics, as it requires a combination of phagocytosis and locomotion. In addition, Albrecht-Buehler (1) also reported that different cell lines produced different particle-free tracks on gold particle-coated substrates that we also readily see with A549 and H322 cells using the BCSM assay (Figures 1 and 2).

Unlike the phagokinetics technique described previously (1) where dishes were coated with gold particles in the presence of BSA, the preparation of our ink-coated dishes did not include BSA (5% FBS was only added to the DMEM culture medium for A549 and H322 cell lines). This could be of significant advantage for cells whose medium must not contain any FBS or BSA, as is the case for HBE-E6E7 and normal bronchial epithelial cells (2), because it might influence their motility. Finally, the concentration of ink particles in PBS solution used in the BCSM assay does not affect viability or motility.

In conclusion, cell-cell interaction constitutes a basic process in the formation of tissues and organs in multicellular organisms. Defects in this phenomenon are involved in various pathological processes, including the development of neoplastic and invasive carcinomas. We propose a simple, inexpensive and highly efficient method to study cell spreading and motility in vitro.

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Figure 2. The BCSM assay in A549 cells. Particle-free tracks produced by these cells 24 h after plating without any treatment. The quantification of this auto-motility revealed that cells cleared ink particles (arrows) over an area of 200.7 µm²/24 h on average per cell.