APPLICATION NOTE

Measuring E-cadherin localization in HT-29 human colon cancer cells

INTRODUCTION

E-cadherin is a calcium-dependent cell adhesion molecule that supports the integrity of epithelial tissues. E-cadherin extracellular domains bind other E-cadherins on neighboring cells, while the intracellular domains bind the cytoskeleton via catenins and other proteins.[1] E-cadherin-based adherens junctions coordinate with actin, myosin, and microtubules to regulate epithelial mechanical properties during embryonic development and adult tissue homeostasis.[2-4] These junctions also interact with multiple signaling pathways to regulate cell cycle progression, differentiation, and epithelial barrier permeability.[4]

E-cadherin acts as a tumor suppressor by imposing physical constraints on cell proliferation and migration and by interacting with signaling molecules to maintain the epithelial phenotype and inhibit cell proliferation.[5] Many cancers downregulate E-cadherin expression, releasing catenins to the cytoplasm and nucleus and enabling tumor cell proliferation, epithelial-mesenchymal transition, and metastasis (Figure 1). E-cadherin loss is associated with advanced tumor stage and poor cancer prognosis.[5,6] E-cadherin overexpression in mouse breast cancer xenografts can prevent tumor progression and decrease tumor size.[7] Therefore, compounds that increase E-cadherin membrane localization have the potential to treat cancer by restoring cell-cell adhesion and inhibiting cancer progression.

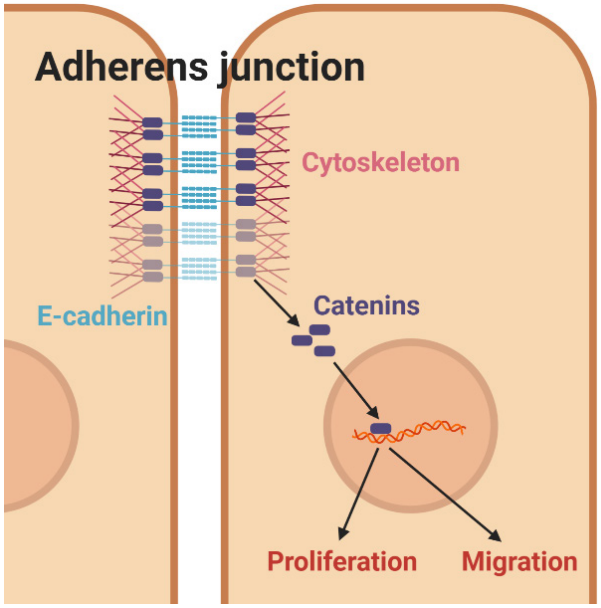

Figure 1. *E-cadherin-mediated cell-cell adhesion at the adherens junction. E-cadherin extracellular domains bind to E-cadherins on neighboring cells, while intracellular domains bind to the cytoskeleton via catenins. Loss of E-cadherin at the membrane releases catenins, which migrate to the nucleus and change gene expression to stimulate cell proliferation and migration. Figure made with Biorender.com.*

In this pilot study, we tested if Vala's IC200 automated high-throughput digital microscopy workstation and CyteSeer® cell image analysis software can detect changes in E-cadherin localization in HT-29 colon cancer cells. To capture high-resolution information on E-cadherin localization, we used Vala's Structured Illumination Microscopy (SIM™), which produces confocal-equivalent resolution with high throughput and low phototoxicity. We tested the effects of phorbol 12-myristate 13-acetate (PMA), which activates protein kinase C signaling and upregulates E-cadherin membrane localization in A431 squamous carcinoma cells[8], and nocodazole, which inhibits microtubule dynamics. Our results demonstrate that Vala's high content screening technology can quantify E-cadherin expression and localization in hundreds of cells per well, enabling high-throughput screening for new cancer therapies.

METHODS

HT-29 human adenocarcinoma colorectal cancer cells (American Type Culture Collection [ATCC]) were thawed per the manufacturer's suggested protocol and maintained at 37° and 5% CO_2. Prior to compound treatment, cells were plated in 384-well plates with optically clear well bottoms at a density of 3,000 cells per well in a total volume of 25 µl. Twenty-four hours after cell plating, one group of wells was treated with 0.1% DMSO alone or 100 nM or 300 nM PMA dissolved in DMSO for 30 minutes. A second group of wells was treated with 0.1% DMSO alone or 250 ng/ml or 500 ng/ml of nocodazole dissolved in DMSO for 24 hours. Six wells were treated per condition.

Following treatment, cells were fixed and immunostained for E-cadherin using a mouse anti-E-cadherin primary antibody targeted to the intracellular domain (BD Biosciences Cat. No. 610181) and a goat anti-mouse AlexaFluor488 secondary antibody (Invitrogen). Nuclei were stained with Hoechst 33342 (Invitrogen). The cells were imaged using Vala's IC200 SIM™, which navigated to each well and acquired four contiguous images in a 2x2 array with a 20x objective. The instrument acquired a z-stack with a range of 5 µm and 1 µm steps and used the SIM Synthetic Focus feature to compile images with optimal focus.

E-cadherin expression analysis was performed using Vala's CyteSeer® automated image analysis software. CyteSeer® identified, segmented, and indexed each cell in each field of view based on nuclei staining to allow for cell-by-cell reporting of measurements. E-cadherin labeling on the plasma membrane was used to guide membrane segmentation and report E-cadherin average pixel intensity at the membrane for each cell. CyteSeer® also identified E-cadherin cytoplasmic puncta and calculated their number, area, and total intensity.

RESULTS

CyteSeer® accurately identified the plasma membranes at HT-29 cell-cell junctions from the E-cadherin immunostaining (Figure 2). CyteSeer® generated a membrane mask by identifying bright, near-continuous lines of E-cadherin around each cell. In regions where E-cadherin-positive membranes from neighboring cells touch, CyteSeer® expanded the mask to include all nearby E-cadherin signal. Regions of E-cadherin membrane staining that did not touch other cells (e.g., at the borders of cell groups) were excluded so that the final membrane mask included only E-cadherin at cell-cell junctions (Figure 2B).

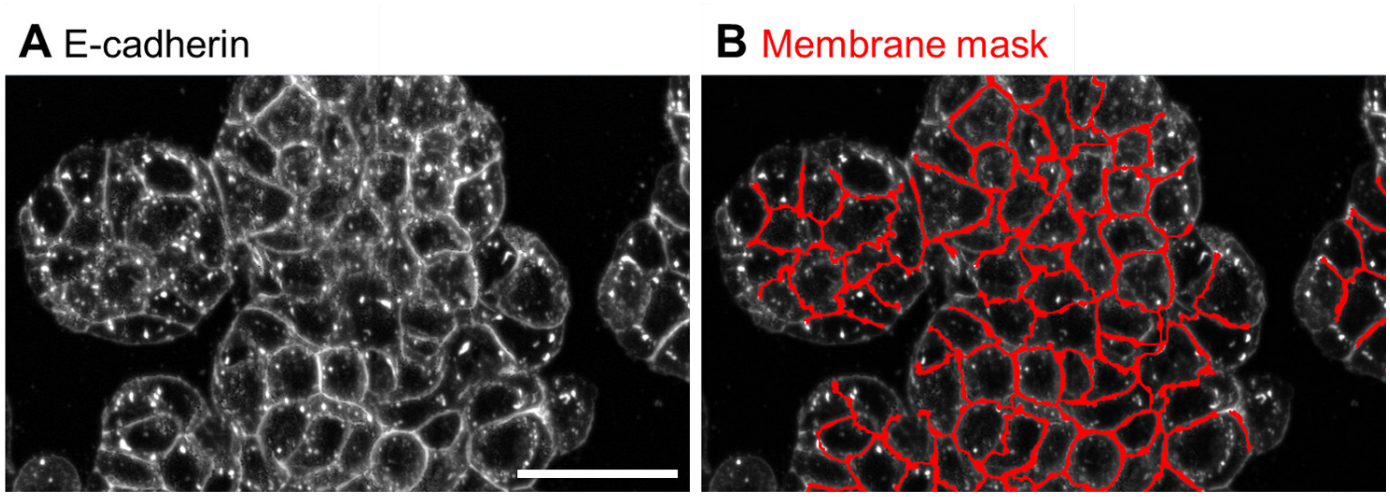

***Figure 2**. E-cadherin-guided membrane segmentation by CyteSeer®. (A) Image of HT-29 cells treated with DMSO alone and labeled with anti-E-cadherin primary antibody and AlexaFluor488 secondary antibody. E-cadherin localizes to the plasma membrane at cell-cell junctions and to puncta in the cytoplasm. (B) Membrane mask identified by CyteSeer® from E-cadherin staining at cell-cell junctions (red). Scale bar: 50 µm.*

To quantify E-cadherin localization to cell-cell junctions, CyteSeer® calculated the average E-cadherin pixel intensity across the membrane mask for each cell. Treatment with 100 or 300 nM PMA for 30 minutes increased E-cadherin cell-cell junction localization by 25% and 29% respectively compared to DMSO alone (Figure 3A-C). These results are consistent with a previous study by Vala researchers in which exposure to 100 nM PMA upregulated E-cadherin at the membrane of A431 cells, a squamous carcinoma cell line, by 30%.[8]

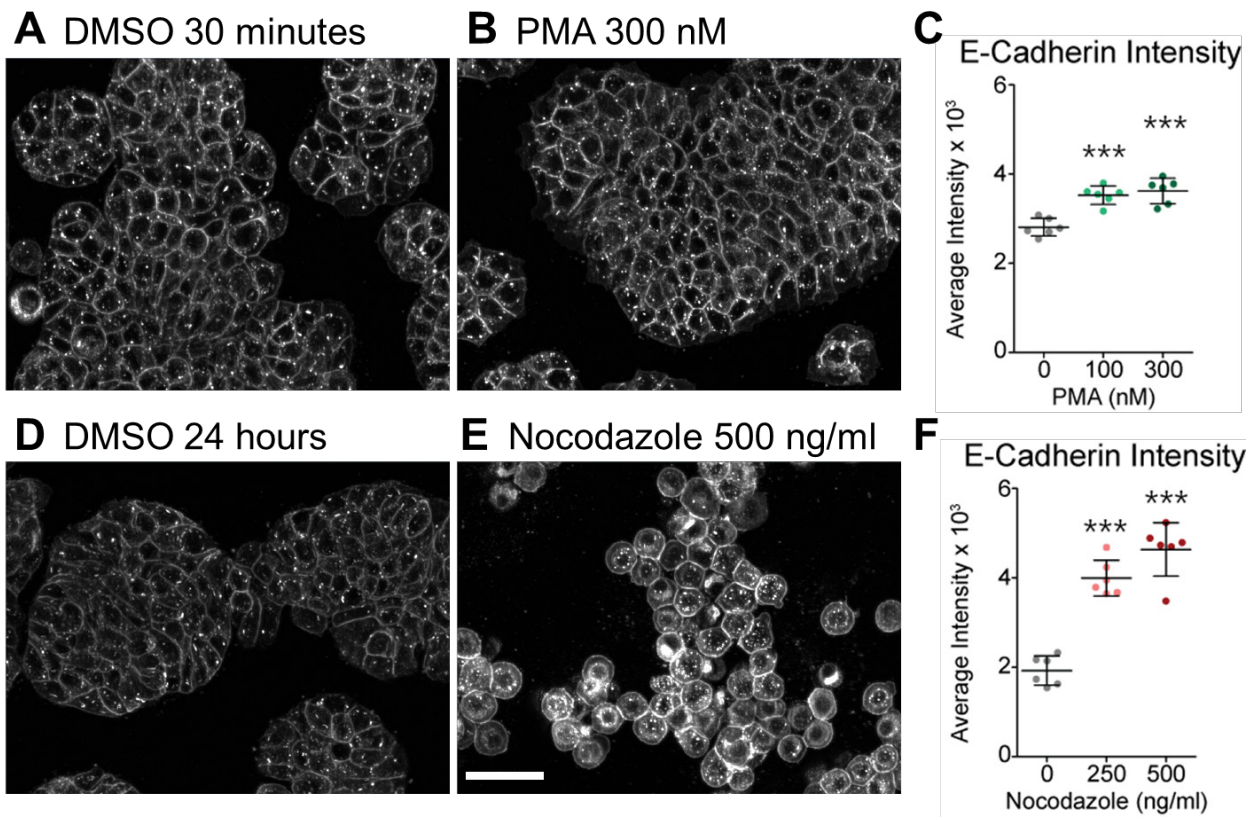

Figure 3. *PMA and nocodazole increase E-cadherin localization to the membrane at cell-cell junctions. (A,B) Representative images of HT-29 cells treated with DMSO or 300 nM PMA for 30 minutes. (C) Dose-dependent increase in E-cadherin membrane localization following PMA treatment. (D,E) Representative images of HT-29 cells treated with DMSO of 500 ng/ml nocodazole for 24 hours. (F) Dose-dependent increase in E-cadherin membrane localization following nocodazole treatment. Each dot represents the mean of each value across all cells in each well, n = 6 wells per condition. Bars and error lines represent mean ± SD. Statistical analysis by ANOVA followed by Dunnett's post-test; ***p<0.001. Scale bar: 50 µm.*

Treatment with 250 or 500 ng/ml of nocodazole for 24 hours increased E-cadherin cell-cell junction localization 107% and 140% respectively compared to DMSO alone (Figure 3D-F). These results suggest that HT-29 cells respond to nocodazole differently from other cell types, in which nocodazole downregulates E-cadherin at cell-cell junctions.[3] Nocodazole-treated HT-29 cells had a rounded shape and had bright E-cadherin puncta in the cytoplasm (Figure 3E).

E-cadherin localized to puncta throughout HT-29 cells. At the membrane, these puncta may represent clusters of E-cadherin at adherens junctions. In the cytoplasm, these puncta may represent endocytosed E-cadherin and/or E-cadherin C-terminal fragments. To quantify E-cadherin endocytosis and proteolysis, CyteSeer® identified cytoplasmic puncta and calculated their number, area, and intensity (Figure 4). While treatment with PMA did not affect E-cadherin cytoplasmic puncta, treatment with nocodazole increased their number, total area, and total intensity (Figure 5). These results demonstrate that CyteSeer® can accurately make multiple measurements of protein localization from each image for hundreds of cells in each well.

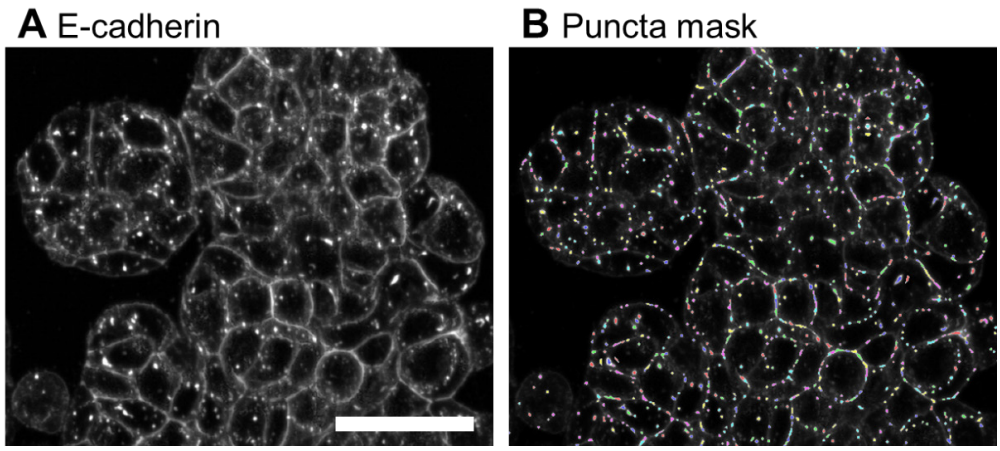

Figure 4. *Segmentation of E-cadherin puncta by CyteSeer®. (A) Image of HT-29 cells labeled with anti-E-cadherin primary antibody and AlexaFluor488 secondary antibody. E-cadherin puncta localize to the plasma membrane at cell-cell junctions and to the cytoplasm. (B) E-cadherin puncta identified by CyteSeer® from E-cadherin staining. Each puncta is assigned a unique color. Scale bar: 50 µm.*

DISCUSSION

Gastrointestinal cancers like colorectal cancer have low survival rates and account for more than one third of cancer-related deaths.[9] Therapeutic strategies that increase E-cadherin localization to cell-cell junctions to inhibit tumor cell proliferation and migration have the potential to improve prognosis for this deadly disease. Our results demonstrate two mechanisms which could lead to such effects: disruption of microtubule dynamics and activation of protein kinase C. Microtubule-disrupting agents such as Taxol effectively treat cancer, and this compound class is currently

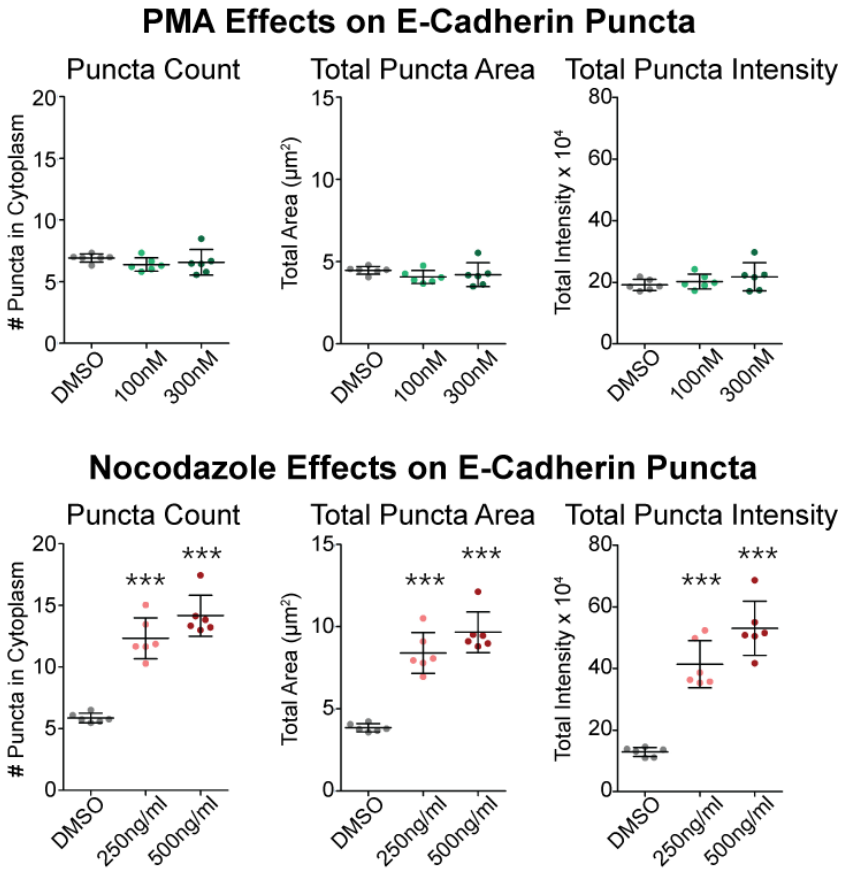

Figure 5. *Nocodazole, but not PMA, increases E-cadherin puncta count, area, and intensity in the cytoplasm. Graphs show the number of E-cadherin puncta identified in the cytoplasm (left), the total area of all cytoplasmic puncta (center), and the total integrated intensity of all cytoplasmic puncta (right) following PMA treatment (top) or nocodazole treatment (bottom). E-cadherin puncta inside the membrane mask (Figure 2) were excluded. Each dot represents the mean of each value across all cells in each well, n = 6 wells per condition. Bars and error lines represent mean ± SD. Statistical analysis by ANOVA followed by Dunnett's post-test; ***p<0.001.*

being explored to identify additional chemotherapeutic agents.[10] The protein kinase C family has both oncogenic and anti-oncogenic roles in cancer biology.[11] Prolonged PMA exposure (5 hours) downregulates E-cadherin in HT-29 cells[12], as opposed to the upregulation following 30 minutes we observed in this study. Our results demonstrate that Vala's instrumentation (Structured Illumination Microscopy™) and image analysis software (CyteSeer®) can quantify compound effects on E-cadherin expression, localization to cell-cell junctions, and intracellular trafficking to increase our understanding of the mechanisms that regulate this important adhesion protein and accelerate the search for anti-cancer agents.

CONCLUSIONS

The E-cadherin localization assay presented here can be expanded to include other cadherins (e.g., N-cadherin), catenins (e.g., β-catenin), and/or signaling molecules. CyteSeer® can use high-resolution Structured Illumination Microscopy™ images to precisely quantify protein localization to the membrane, cytoplasm, and nucleus on a cell-by-cell basis without the need for cell fractionation or tissue dissociation. High content screens on epithelia-derived cancer cells using Vala's technology can increase the likelihood of discovering effective chemotherapeutics. Previous research has applied Vala's high content screening technology to quantify perilipin localization to lipid droplets in human and murine preadipocytes[13] and transcription factor expression during pancreatic regeneration in response to injury.[14] Thus, Vala provides a versatile automated microscopy workstation with broad applications across biomedical and cell biology research.

CONTACT US

Please contact Vala Sciences, Inc at www.valasciences.com/contact, info@valasciences.com, or toll-free at (858) 742-8252 for more information about purchasing an instrument, which includes a copy of CyteSeer®. We also provide drug discovery or safety screening services using our technologies.

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