Technical Bulletin #441

Screening of Anti-Metastatic Compounds Using ZsGreen1 Reef Coral Fluorescent Protein (RCFP) Labeled HT-1080 Tumor Cells

[1]*Paula Flaherty*, [2]*Amy Goldberger, Ph.D.*, [2]*Frank Mannuzza, Ph.D.*, [2]*Stephen Ilsley*, [3]*Olivier Dery, Ph.D.*, [3]*Brigette Angres, Ph.D.*
[1]*Corning Life Sciences, Tewksbury, MA USA;* [2]*BD Biosciences, Billerica, MA USA;* [3]*Clontech - a Takara Bio Company, Palo Alto, CA USA*

CORNING

Please note: the data generated in this Technical Bulletin (TB) used the original (purple) FluoroBlok™ membrane. We have made improvements to this membrane since it was introduced. The new FluoroBlok uses a black membrane with improved spectral characteristics. General information in this TB applies to both versions of the product, but the specific wavelength ranges mentioned here apply only to the original (purple) version.

For details, see Technical Bulletin **CLS-DL-CC-042**: *New PET Membrane for Corning FluoroBlok 3.0 µm and 8.0 µm Pore Size Cell Culture Inserts.*

Introduction

The development of fluorescent labeling technology has provided an innovative tool for measuring dynamic processes of the living cell *in vitro*. Green Fluorescent Protein (GFP), from the luminescent jellyfish Aequorea victoria, has augmented traditional cell detection methods by providing an inherent, autocatalytic fluorophore that does not require exogenous cofactors. We have incorporated the GFP homolog ZsGreen1, a fluorescent protein from the nonbioluminescent Indo-Pacific reef coral *Zoanthus sp.* (Reef Coral Fluorescent Protein, RCFP), into the human fibrosarcoma cell line, HT-1080.

HT-1080 cells together with the reconstituted basement membrane Corning® Matrigel® Matrix, derived from the Englebreth-Holm-Swarm (EHS) mouse tumor, provide a robust, functional model for the multi-factorial cascade of tumor metastasis. The tumor cell must dissociate from the primary mass, migrate through the interstitial extracellular matrix, invade the basement membrane underlying the vascular endothelium and enter the circulation. The tumor cell must then attach to and invade through the endothelial cell layer and basement membrane to enter the interstitial matrix and establish a metastasis. Corning Matrigel Matrix, coated on cell culture inserts, emulates the critical barrier of the basement membrane to the passage of tumor cells *in vivo*.

The use of a dyed microporous PET membrane multiwell insert which blocks the transmission of light from 490-700 nm allows for the detection of the RCFP HT-1080 cells on the underside of the membrane after invasion through Corning Matrigel Matrix. This methodology simplifies the quantitation of cells, allowing for a homogeneous assay facilitating the screening of anti-metastatic compounds.

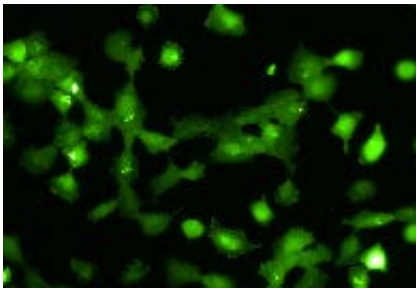
The rate of invasion through a basement membrane as well as migration (without a basement membrane) of non-labeled HT-1080 cells with that of RCFP-labeled HT-1080 cells were compared. This rate study showed that RCFP had no effect on the invasion or migration of HT-1080 cells when compared to non-labeled cells. We then compared the inhibition of invasion and migration by known chemotherapeutic compounds. IC_{50} data was compared for Doxycycline (DOXY), a synthetic tetracycline analog, Paclitaxel, an inhibitor of microtubule disassembly, and 1,10-Phenanthroline, a zinc chelator and metalloprotease (MMP) inhibitor. In all cases the predicted IC_{50} for RCFP HT-1080 cells were analogous to the non-labeled HT-1080 cells.

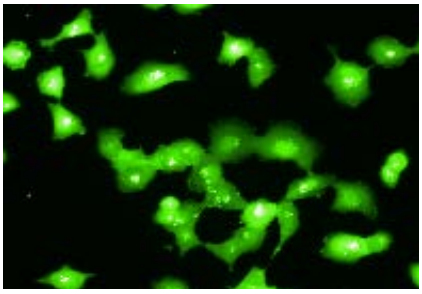
Advances in the technology of fluorescent membrane-based cell assays and in GFPs combine to provide a homogeneous assay for the screening of compounds which alter the invasive phenotype of tumor cells *in vitro*.

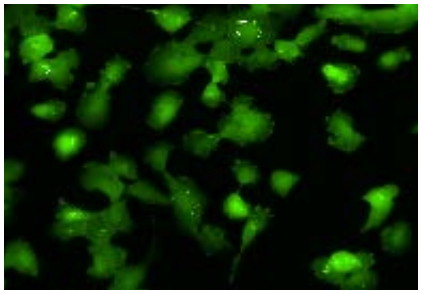
Materials and Methods

Corning FluoroBlok Multiwell Insert System

The 8.0 µm pore size (Cat. Nos. 351157 and 351158) consists of a cell culture insert containing a fluorescence blocking microporous PET membrane. This membrane blocks the passage of light at wavelengths 490-700 nm at greater than 99% efficiency, thus separating the light emitted by fluorescent labeled cells on the upper surface of the membrane from that on the lower surface allowing for real-time, non-destructive monitoring and quantitation of invading or migrating cells without further manipulation. The 24 insert wells are contained within a single unit facilitating manual and automated handling.







Corning® Matrigel® Matrix

Corning Matrigel Matrix is a biologically active reconstituted extracellular matrix preparation derived from the Englebreth-Holm-Swarm (EHS) mouse tumor. Corning Matrigel Matrix coated on the membrane functions as a barrier to the passage of non-invasive cells analogous to the *in vivo* extracellular basement membrane.

Corning BioCoat™ 24-Multiwell Tumor Cell Invasion System

This system (Cat. Nos. 354165 and 354166) was prepared by coating the membrane of a Corning FluoroBlok™ 24-Multiwell Insert (8.0 µm pore size) with Corning Matrigel Matrix. The coating was dried under unique conditions. This procedure results in occlusion of the membrane pores.

HT-1080 Human Fibrosarcoma Cells

HT-1080 cells are a commonly used invasive tumor cell and were obtained from American Type Culture Collection (ATCC).

Corning BioCoat Collagen I Cellware

Collagen I-coated flasks were used.

Media and Additives

Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Glutamine, Penicillin, Streptomycin, Trypsin and G418 Sulfate were obtained from Life Technologies.

Reagents

Calcein AM (C-3100) was obtained from Molecular Probes. DOXY, Paclitaxel and 1,10-Phenanthroline were obtained from Sigma. Corning Fluorescent Dyes are available for labeling cells when performing tumor cell invasion, angiogenesis assays, and other cell-based assays. For additional information about Corning Calcein AM (Cat. Nos. 354216, 354217) visit www.corning.com/lifesciences.

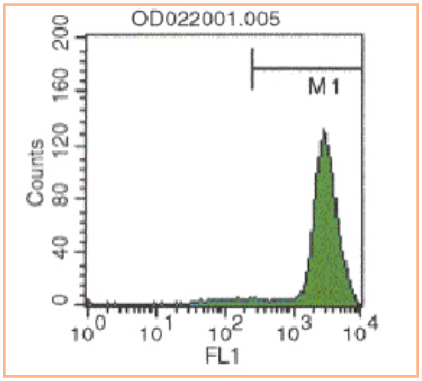


***Figure 1**: BD FACS™ analysis of the RCFP HT-1080 cells.*

Establishment of HT-1080 Cells Expressing ZsGreen1

A retroviral expression system was used to express ZsGreen1 in HT-1080 cells. The cDNA for ZsGreen1 was subcloned in the vector pLNCX2 (Clontech - a Takara Bio Company). The packaging cell line GP2-293 was co-transfected with pVSVG and the pLZsGreen1 construct. HT-1080 cells were then infected for eight hours with the virus particles and put in selective medium for two weeks to select cells expressing the neomycin resistance and the ZsGreen1. Cells were then individually sorted in 96-well plates using a BD FACSVantage™ and clones were grown for about ten days. Expression of ZsGreen1 was assessed by flow cytometry and fluorescent microscopy. ***Figure 1*** shows the FACS analysis of the RCFP HT-1080 cells. As an alternative cell reagent that is suitable for studying tumor cell migration and invasion, HT-1080 cells have been stably transfected with the DsRed-Express Fluorescent Protein (HT-1080 Retro DsRed-Express Cell Line, Cat. No. 632454, Clontech - a Takara Bio Company).

Preparation of Cell Suspensions

HT-1080 and RCFP HT-1080 cells were grown to near confluence in DMEM containing 10% FBS. RCFP cell medium was supplemented with 2 mM glutamine, 50 units/ml penicillin, 50 µg/ml of streptomycin and 0.5 mg/ml of G418 Sulfate. Cell suspensions were prepared by trypsinizing the monolayer and resuspending in DMEM without serum.

RCFP HT-1080 cells were grown on Corning BioCoat Collagen I-coated flasks. Cells exhibited attachment and spreading on collagen-coated flasks and a spindle shape on uncoated plastic. When seeded at the same cell density collagen-coated flasks increased cell yield by 2x when compared to uncoated flasks.

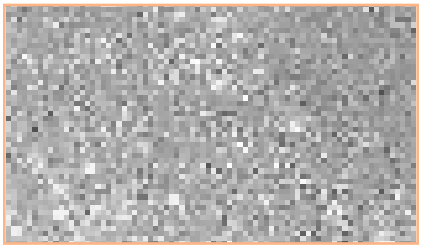


***Figure 2**: RCFP HT-1080 cells grown on Corning BioCoat Collagen I-coated flasks.*

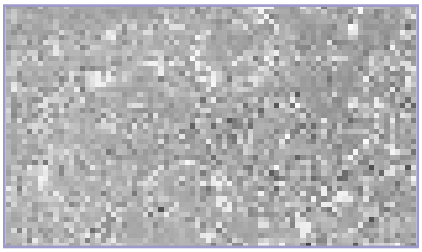


***Figure 3**: RCFP HT-1080 cells grown on uncoated plastic.*

Figure 2 demonstrates the spreading and attachment of RCFP HT-1080 cells on Corning BioCoat Collagen I-coated flasks. ***Figure 3*** shows the spindle morphology on uncoated plastic. Cells were seeded onto Corning BioCoat Collagen I 175 cm^2 flasks at 3 x 10^5 and harvested two days later. Cells grown on collagen I-coated flasks yielded 8.5 x 10^5, while those grown on uncoated plastic yielded 4.6 x 10^5.

Invasion Assay Procedure

The assay insert plates were prepared by rehydrating the Corning Matrigel Matrix coating with phosphate buffered saline for two hours at 37°C. The rehydration solution was carefully removed, 0.75 ml DMEM containing chemoattractant (5% FBS) was added to the plate well and 0.5 ml of cell suspension (5 x 10^4 cells/ml, 2.5 x 10^4 cells/insert) was added to each insert well. For invasion inhibition assays, the compound of interest was added to the medium in both the upper and lower chambers. Uncoated insert plates, included as migration controls, were used without rehydration. Assay plates were incubated for 20-22 hours at 37°C, 5% CO_2.

RCFP plates were read in a fluorescent plate reader without further manipulation.

HT-1080 Plates

Medium was removed from the upper chamber and the entire insert plate was transferred to a second 24-well plate containing 0.5 ml/well of 4 µg/ml calcein AM in Hanks buffered saline. The plates were incubated for one hour at 37°C and read in a fluorescence plate reader.

96-well Migration Assay Comparing Calcein AM and RCFP HT-1080 Cells

HT-1080 human fibrosarcoma cells were grown to 60-80% confluence in T-75 flasks. The media was removed, and the cells were labeled for one hour with 10 µM calcein AM in serum-free DMEM at 37°C, 5% CO_2. The cells were then briefly trypsinized in 0.5% trypsin-EDTA, washed and resuspended in DMEM/0.1% BSA. RCFP-expressing HT-1080 cells were grown to 50-70% confluence on Corning BioCoat Collagen I 175 cm^2 flasks. Cells were trypsinized, washed and resuspended in DMEM/BSA. Both the calcein-labeled and the RCFP-expressing cells

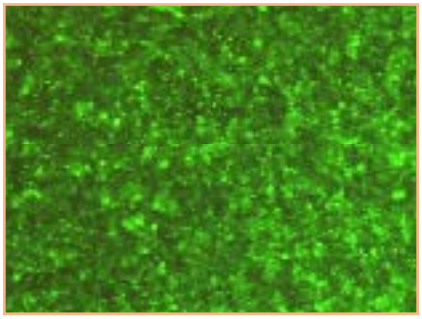


Figure 4: *RCFP HT-1080 cells on the bottom of the Corning® FluoroBlok™ insert, post-invasion.*

were counted and cell viability was determined using trypan blue.

After dilution in DMEM/BSA to give 2,000-16,000 cells in 50 ml, the cells were placed in the inserts of a prototype 8.0 µm Corning FluoroBlok 96 Multiwell Insert Plate. The wells of the receiver plate contained either DMEM/BSA or DMEM/5% serum as the chemoattractant. Assays were incubated at 37°C, 5% CO_2 and quantitated at the indicated times in a fluorescence plate reader set in bottom-reading mode. Data represents the mean of n=8 wells ± SD.

Standard Curve

Serial dilutions of the cell stocks (100-100,000 cells/well) were dispensed into 96-well plates and quantitated on the fluorescence plate reader to give a standard curve. Data represents the mean of n=8 wells ± SD. The standard curve was used to convert mean fluorescent values from the corresponding migration assays into cell numbers.

Fluorescence Reading

Fluorescence data was collected using an Applied Biosystems CytoFluor™ 4000 Fluorescence Multiwell Plate Reader at excitation wavelength (ex) of 485 nm and emission wavelength (em) of 530 nm at a gain of 55 nm. Only those labeled cells that pass through the Corning Matrigel® Matrix layer and the membrane are detected.

Data Analysis

IC_{50} values were calculated using either the classical four parameter logistic model or the nonparametric smoothing spline.

Results and Discussion

Methods of screening potential cancer therapies directed against tumor metastasis have not kept pace with other rapid technological advancements in drug discovery. Measurement of tumor cell invasion through a microporous membrane coated with Corning Matrigel Matrix in Boyden-like chambers is a widely accepted *in vitro* assay emulating the critical barrier of the basement membrane in metastasis. However, these methods require arduous manipulation of samples resulting in wide variations in data.

The development of fluorescent technologies has provided an innovative tool for measuring functional activities of cells *in vitro*. We have combined a standard tumor cell invasion assay with a dyed PET membrane that blocks the transmission of light from 490-700 nm, separating light emitted by fluorescent-labeled cells on the upper surface of the membrane from that on the lower, allowing quantitation of invading or migrating cells. In addition we have transfected a model invasive cell line, the human fibrosarcoma HT-1080, with ZsGreen1, a fluorescent protein from the reef coral *Zoanthus sp*. The result is a single step, homogeneous assay for screening anti-invasive compounds.

In order to demonstrate that RCFP HT-1080 cells are a useful tool in predicting IC_{50}s of anti-invasive compounds we first demonstrated that the cells remain invasive after transfection with the ZsGreen1 protein (62% of migrating RCFP cells invade) (***Figure 5***). This corresponds with invasion of unlabeled HT-1080 cells (60-80%). In a 24-hour assay, RCFP cells invaded at 92% and migrated at 90% of unlabeled cells (***Figure 6***). This 8-10% drop in migration/invasion may indicate that exogenous expression of fluorescent labels can effect cellular function. However, the effect is not enough to discount the use of these cells for compound screening. Standard curves run in 96-well plates indicate a sensitivity to as low as 100 cells (***Figure 13***). Using Corning FluoroBlok 96-Multiwell Insert Plates (Cat. Nos. 351163 and 351164), 8.0 µm pore size, RCFP-labeled cells lagged behind calcein AM-labeled cells in a short-term (four-hour) assay. Migration was slowed, but at 21 hours had recovered (***Figure 14***). This indicates endogenous expression of fluorescent labels can affect cellular functions.

Three anti-invasive compounds were chosen to demonstrate the effectiveness of the RCFP cells in predicting IC_{50}s. DOXY is a synthetic tetracycline analog and antimicrobial agent. It has been shown to suppress tumor metalloproteinase (MMP) production and function as a non-competitive inhibitor. Paclitaxel is an inhibitor of disassembly of microtubules and is an established chemotherapeutic agent. 1,10-Phenanthroline is a zinc chelator and metalloproteinase inhibitor which has been shown to prevent tumor cell invasiveness, as well as endothelial cell invasion, an important event occurring during angiogenesis.

Compounds were tested with unlabeled HT-1080 cells and then compared to data collected for RCFP cells. Invasion through a basement membrane and migration through an uncoated PET membrane were inhibited by each compound. Predicted IC_{50}s for both migration and invasion gave results in the expected range (***Figures 7-12***). Though label intensity for RCFP was lower than that for calcein AM, the signal to background allowed for discrimination of concentration curves and accordingly, the prediction of IC_{50}s.

We have effectively combined advances in PET membranes and in fluorescent technologies to develop a single-step homogeneous invasion assay.

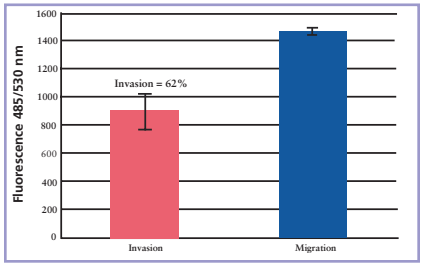


Figure 5*: In order to test capability to invade a basement membrane and the fluorescent cell signal to background, RCFP HT-1080s were assayed for invasion through a Corning Matrigel Matrix-coated Corning FluoroBlok membrane and migration through an uncoated membrane. After 24 hours, cells on the bottom of the insert were read on a fluorescent plate reader. RCFP cells invaded through Corning Matrigel Matrix at 62% which is consistent with previous data for unlabeled HT-1080 cells.*

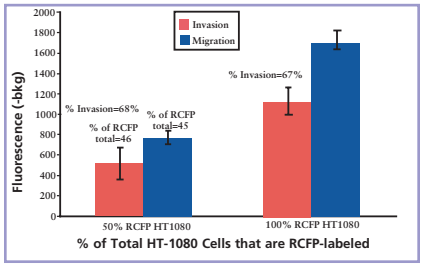
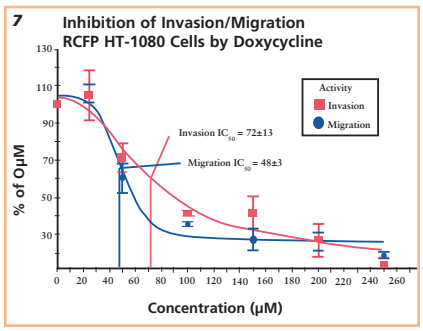
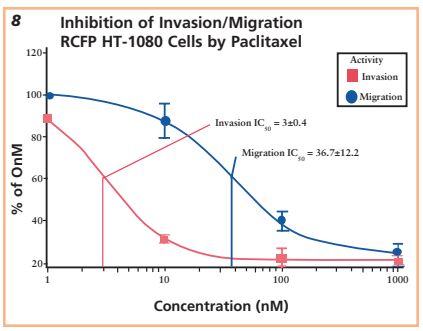
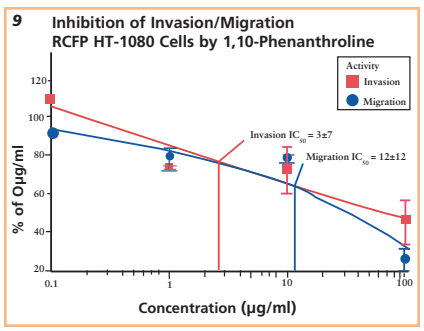
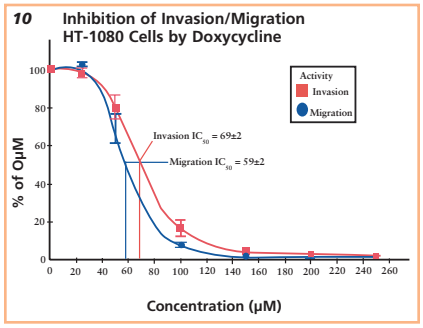


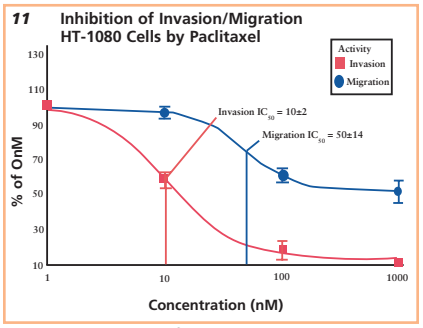
Figure 6*: To compare the rate of invasion/migration of RCFP with unlabeled HT-1080 cells, Corning FluoroBlok inserts were seeded with either all RCFP HT-1080 cells or with a mixture of 50% RCFP and 50% unlabeled HT-1080 cells. The rate of invasion of RCFP cells at 24 hours was at 92% of the unlabeled HT-1080 cells. Migration of the RCFP cells was at 90% of the unlabeled cells. The percent of cells that invade when compared to migration of cells is consistent with previous data at 67-68%.*

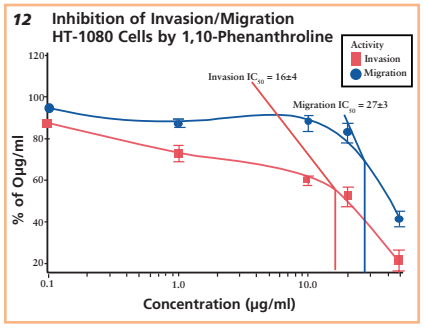












***Figures 7-12**: RCFP labeled HT-1080 cells were used to predict the IC_{50} of three anti-invasive compounds. These IC_{50} data were compared to those predicted by unlabeled HT-1080 cells. In each case the data for invasion and migration predicted for unlabeled HT-1080 cells was emulated by the IC_{50} data predicted by the RCFP HT-1080 cells.*

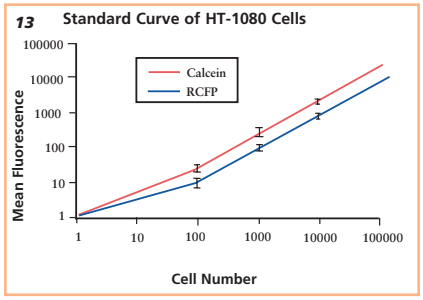


***Figure 13**: To determine the limits of detection, RCFP HT-1080 cells were compared to calcein AM-labeled cells in a standard curve. Detection was linear down to 100 cells/well and 1,000 cells/well could easily be detected.*

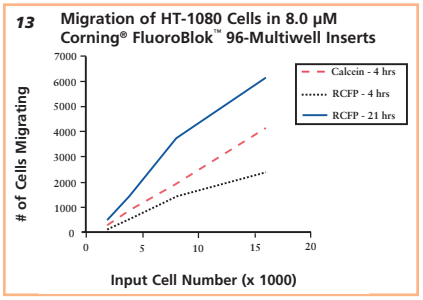


***Figure 14**: Demonstrating the utility of the RCFP cells in a smaller format, a prototype of the Corning FluoroBlok 96-Multiwell Insert was used to study migration. Titration of the input cell number showed the migration of about 1,000 cells could be detected in four hours when starting with 8,000 cells, this number tripled after 21 hours. In a short-term (four-hour) assay, migration of RCFP cells lagged behind that of calcein AM-labeled cells, at 21 hours. However, RCFP-labeled cells overcame this lag. The relationship between input cell number and the number of cells migrating was linear for calcein AM-labeled cells but leveled off at higher input cell numbers for the RCFP-expressing cells.*

Corning acquired the BioCoat™, FluoroBlok™, and Matrigel® brands.
For information, visit www.corning.com/discoverylabware.

CORNING

Corning Incorporated
Life Sciences
836 North St.
Building 300, Suite 3401
Tewksbury, MA 01876
t 800.492.1110
t 978.442.2200
f 978.442.2476
www.corning.com/lifesciences