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A paper-based invasion assay: Assessing chemotaxis of cancer cells in gradients of oxygen

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ABSTRACT

This work describes a 3D, paper-based assay that can isolate sub-populations of cells based on their invasiveness (i.e., distance migrated in a hydrogel) in a gradient of concentration of oxygen (O_2). Layers of paper impregnated with a cell-compatible hydrogel are stacked and placed in a plastic holder to form the invasion assay. In most assays, the stack comprises a single layer of paper containing mammalian cells suspended in a hydrogel, sandwiched between multiple layers of paper containing only hydrogel. Cells in the stack consume and produce small molecules; these molecules diffuse throughout the stack to generate gradients in the stack, and between the stack and the bulk culture medium. Placing the cell-containing layer in different positions of the stack, or modifying the permeability of the holder to oxygen or proteins, alters the profile of the gradients within the stack. Physically separating the layers after culture isolates sub-populations of cells that migrated different distances, and enables their subsequent analysis or culture. Using this system, three independent cell lines derived from A549 cancer cells are shown to produce distinguishable migration behavior in a gradient of oxygen. This result is the first *experimental* demonstration that oxygen acts as a chemoattractant for cancer cells.

1. Introduction

Hypoxia—the reduction of levels of oxygen tension to values below normal in a tissue—is toxic to cells [1,2]. Cancer cells exposed to hypoxia, both *in vitro* and *in vivo*, undergo genetic and/or phenotypic changes that influence cellular metabolism, proliferation, and development of radio/chemo-resistance [3–6]. These changes allow the cells to survive in stressful environments. Gradients in concentration of oxygen (we use "oxygen" to mean O_2) as well as other small molecules (e.g., glucose, nutrients, signaling factors, cellular waste products) [1–4,6] develop normally in tissue due to competition between the supply of oxygen (a process dictated by mass transport) and its consumption by cellular metabolism. These gradients span ~180 μm radially from a typical blood vessel. Gradients also develop in solid tumors, but are steeper than those formed in normal tissue due to poorly developed and distributed blood vessels.

Mathematical models have predicted that gradients of oxygen within a tumor direct the migration of cancer cells from the primary tumor to surrounding tissue [5], but to date there has been limited experimental evidence against which to compare these models. This lack of experimental data is due largely to the difficulty in controlling and measuring gradients of oxygen in living tissue, and to the difficulty associated with analyzing the migratory response of cells in conventional model systems (both *in vivo* and *in vitro*) [7–11]. These mathematical models predict that asymmetric gradients of oxygen and other small molecules such as glucose can cause differential rates of proliferation of cells in a

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tumor, and these gradients ultimately contribute to the formation of primary tumors with irregular shapes [5]. The models also predict an asymmetric migration of cells by the budding and the splitting-off of small populations of cells from the primary tumor. The migration of small groups of cells from the primary tumor is termed "collective invasion", and has been observed both *in vivo* and *in vitro* [12–18].

There are a number of methods to assess cellular migration and invasiveness *in vitro* [9–11,19–23]; these methods rely on quantifying movement across two-dimensional (2D) substrates or through three-dimensional (3D) gels. The concentration gradient of small molecules can be controlled temporally and spatially (at length scales of tens of microns, which is the scale of a single cell) in a microfluidic device [7,8,24–28]. These devices are often fabricated in optically transparent materials and make it possible to visualize cell migration in real time [19]. Despite the experimental control offered by a microfluidic device, commonly used invasion assays—such as the Transwell assay (otherwise known as a Boyden chamber), or a Dunn chamber—sacrifice these precisely defined gradients for ease-of-use, simple end-point readouts, and scalability [9]. It is not possible to form gradients of oxygen in a Transwell assay or in a Dunn chamber, and the migratory behavior of cells in response to hypoxic gradients cannot be studied. While a variety of microfluidic devices have been developed to control precise gradients of concentration of oxygen [7,8,24–29], only recently has a device been used to investigate the migration of cells in such gradients [29]. Tung et al. demonstrated that oxygen acts as a chemorepellant for A549 cells and these cells migrate toward lower concentrations of oxygen in the presence and absence of an overlapping concentration gradient of stromal cell-derived factor 1 (SDF-1); the gradient of SDF-1 was perpendicular to the concentration gradient of oxygen [29].

To determine if gradients of oxygen direct cellular invasion, we developed an *in vitro* assay in which cells are cultured in a 3D construct that enables us to analyze cellular migration within a tissue-like environment (Fig. 1A). Unlike most commonly used invasion assays [9,10,19,22,23,29], which impose a gradient of molecules on cells to direct their movement, this assay mimics the mechanism by which gradients are generated *in vivo* [30–33], and allows the cells to consume and secrete molecules within a diffusion-dominated environment.

A paper-based invasion assay—based on the previously published "Cells-in-Gels-in-Paper", or CiGiP [30–33]—combines the simplicity of commonly used invasion assays with the ability of a microfluidic device to generate gradients of small molecules (albeit with significantly less precision than those generated in a microfluidic device). A unique feature of this assay is the ability to isolate easily cells that migrated different distances by physically separating the layers of the 3D construct (by peeling them apart) after a given period of incubation (Fig. 1). In CiGiP, individual layers of paper are impregnated with cells suspended in a hydrogel and assembled into tissue-like structures by stacking the individual

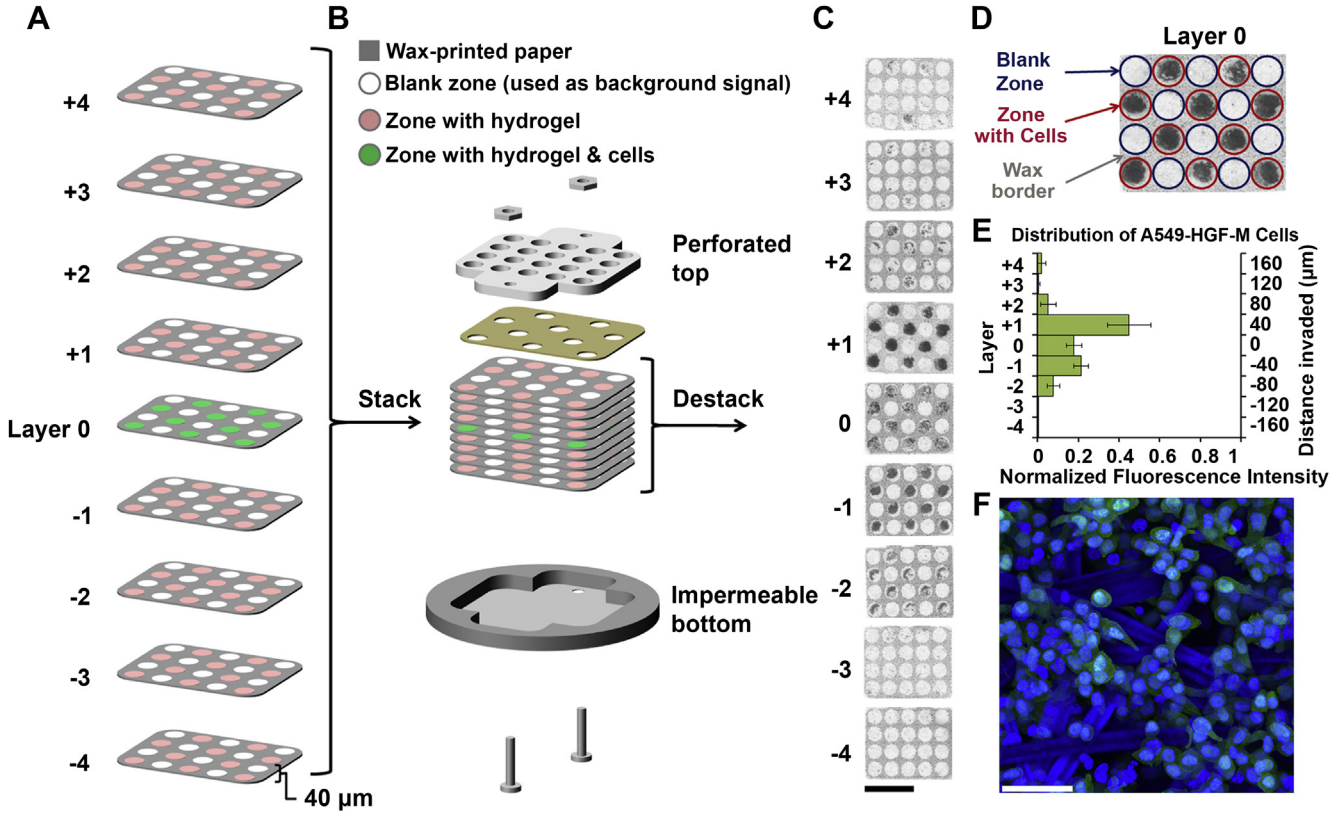

Fig. 1. Paper-based invasion assay. A) Schematic of a single layer of wax-patterned paper (40 μm thick) containing GFP-expressing A549-HGF-M cells suspended in a hydrogel (Layer 0) positioned between sheets of paper containing only hydrogel (Layers $0 + n$ for layers above the seeded layer, and $0 - n$ for layers below the seeded layer). B) Schematic of all layers of paper stacked with a laser-cut transparency in an acrylic holder using two screws and nuts. C) Images from a fluorescent scanner of all layers of paper after 24 h in culture. An array of circular zones is visible due to the autofluorescence of the wax. Within the circles, darker pixels correlate with a higher intensity of GFP (e.g., higher density of cells). Scale bar: 1 cm. D) Image of layer 0 with red circles outlining cell-seeded zones and blue circles outlining blank zones, which are used to determine background intensity of the layer. E) Plot of the average intensity of GFP within each layer, normalized by the total intensity of GFP within all layers of the stack. Each layer was cultured separately overnight and then stacked for 24 h before being destacked and imaged. Error bars represent the standard deviation for 10 replicate zones within the same layer. F) Confocal image of cells within layer 0: green is GFP (A549-HGF-M), blue is DAPI (nuclei). Fibers of the paper are visible due to their autofluorescence under ultraviolet irradiation. Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)