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# Statistical Platform to Discern Spatial and Temporal Coordination of Endothelial Sprouting

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### Abstract

Many biological processes, including angiogenesis, involve intercellular feedback and temporal coordination, but inference of these relations is often drowned in low sample sizes or noisy population data. To address this issue, a methodology was developed to statistically study spatial lateral inhibition and temporal synchronization in one specific biological process, endothelial sprouting mediated by Notch signaling. Notch plays an essential role in the development of organized vasculature, but the effects of Notch on the temporal characteristics of angiogenesis are not well understood. Results from this study showed that Notch lateral inhibition operates at distances less than  $31\mu m$ . Furthermore, combining time lapse microscopy with an intraclass correlation model typically used to analyze family data showed intrinsic temporal synchronization among endothelial sprouts originating from the same microcarrier. Such synchronization was reduced with Notch inhibitors, but was enhanced with the addition of Notch ligands. These results indicate Notch plays a critical role in the temporal regulation of angiogenesis, as well as spatial control, and this method of analysis will be of significant utility in studies of a variety of other biological processes.

## Introduction

Biological systems often demonstrate high variability, and standard approaches to analyze these processes may not provide significant insight. Vascular sprouting, a key step in the angiogenesis processes during development and cancer progression, is a prime example. In recent years, numerous studies have elucidated a qualitative role of Notch signaling in angiogenesis. There are five canonical DSL (Delta, Serrate, LAG-2) Notch ligands: Delta-like 1 (Dl11), Dl13, Dl14, Jagged-1 (Jag1), and Jagged-2 (Jag2). Among them, Dl11, Dl14 and Jag1 are the most prevalent in endothelium [1]. Upon binding to DSL ligands, Notch receptors initiate a series of proteolytic cleavages. First, the A Disintegrin And Metalloprotease (ADAM) family within the juxtamembrane region is cleaved. Cleavage of  $\gamma$ -secretase within the transmembrane region subsequently occurs, releasing the Notch intracellular domain (NICD) that translocates to the cell nucleus.

Recent studies have shown Notch signaling regulates many aspects of vascular biology. Endothelial cell specification into tip and stalk cells is regulated by Notch, with strongest Notch activity observed in the stalk cells [1,2]. Pharmacological and genetic inactivation of Notch causes excessive vessel sprouting and branching [2–9], but the resulting vasculature is often not lumenized or productive in perfusion. The angiogenic studies on Notch have mainly focused on its spatial lateral inhibition. Limited quantification has been done on the temporal effects of Notch on angiogenesis [10,11]. Understanding the temporal aspects of angiogenesis, however, is critical for the development of vascular networks. In particular, communication between endothelial cells is required to temporally coordinate sprouting and pave the way for subsequent fusion of nascent blood vessels.

A number of *in vitro* assays have been used to model angiogenesis, each with its advantages and drawbacks. One assay, the aortic ring assay is useful for studies measuring aggregate proliferation and movements of different cell types, but does not separate simple cell migration from endothelial activation [12, 13]. Two dimensional morphogenesis assays, such as the tube formation assay [14], do not represent the 3D environment. In this study, a sprouting assay using microcarrier beads embedded in a 3D gel [15, 16] was used to monitor the temporal effects of Notch on sprouting angiogenesis. In such an assay, the juxtacrine effects mediated by Notch are isolated, because cells from different microcarriers are disconnected. Furthermore, the high sample number per experiment and the uniform shape of the microcarriers potentially allows automated quantitative analysis for large-scaled experimental data. The spherical structure of the microcarriers also can enable automated detection of sprout formation.

In contrast to previous work that has largely studied sprouting on a population level using collective statistics, this study utilized an intraclass correlation model to infer the temporal synchronization of sprouting events within a microcarrier. Despite the low number of sprouts per microcarrier in the sprouting assay, statistical inference could be drawn by taking advantage of the large number of microcarriers in the experiment. The intraclass correlation model was first developed to analyze family data [17,18], and since then was used in various other contexts [19]. In familial analysis, this model was used to estimate the degree of resemblance among family members with respect to certain characteristics, such as height, test scores, or blood pressure. These effects are often hidden in the collective statistics such as sample mean and variance. Equivalently, similarity of the times of sprout initiation is an indirect measure of the synchronization of sprouting. This model, when combined with automated sprout analysis, is demonstrated here to be powerful in extracting information that is otherwise subtle due to the indistinguishability of noisy aggregate data across experimental conditions.

#### Results

#### Validation of Notch Ligands and Inhibitors

The effects of the Notch ligands and inhibitors were validated by immunostaining for the activated Notch intracellular domain (NCID). Treatment with Jag1 and Dll4 significantly increased the level of Notch activity, whereas addition of DAPT abrogated Notch activity (Fig. 1a).

#### In vitro Endothelial Sprouting Assays

The impact of Notch inhibitors and ligands on the overall level of angiogenesis was analyzed using a well-studied *in vitro* sprouting assay [15, 16]. In the sprouting assay, endothelial cells (ECs) coated on dextran beads formed angiogenic sprouts in fibrin gel. A sprout is defined as a multicellular extension with > 1 connected ECs that are attached to the microcarrier (white arrows in Fig. 1b). The sprouting ratio, defined as the number of sprouts divided by the number of microcarriers, was quantified for various conditions (Fig. 1c). In accordance with previous studies, treatment with Notch inhibitors, DAPT and anti-Dll4 antibody, significantly increases the sprouting ratios [2–4, 20]. In contrast, the administration of Dll4 and Jag1 in the gel and media caused a reduction in sprouting ratios. This reduction was reversed when Notch inhibitors were added simultaneously with the Notch ligands.

#### **Analysis of Sprout Spatial Distribution**

As a validation of Notch-mediated lateral inhibition in this assay, the numbers of sprouts on each bead were counted to estimate the distribution of the number of sprouts on the microcarriers (Fig. 2a). The data showed that DAPT caused a slight reduction in the relative frequency of microcarriers with zero sprouts. Interestingly, the frequency of microcarriers with one sprout was also reduced with DAPT treatment. The frequencies at 2 and 3 sprouts, on the other hand, were increased by DAPT. This result was consistent with the notion that Notch acts as a lateral inhibitor between endothelial cells. When this lateral inhibition was relieved by the addition of DAPT, the relative frequencies of microcarriers with multiple sprouts increased.

In order to spatially characterize and to more directly measure Notch-mediated lateral inhibition, the distances between each pair of sprouts were measured. The two-dimensional composite projections for the 3D stacks were taken, and the distance,  $d_{ij}$ , between each pair of observed sprouts (i, j) along the arc of circular microcarrier was measured. Formally, the distance was measured as  $r\delta\theta$  on the coordinate system defined in Fig. 2b. Fig. 2c illustrated the measurements of the distances between one sprout (red arrow) and all the other sprouts. The distances between all possible pairs of sprouts were recorded. It is important to note that unlike the number of sprouts  $n_s$ , the distances  $d_{ij}$  were measured on the 2D projection due to limited resolution in 3D distances. Fig. S1 shows a simulated scatter plot of the 2D and 3D distances of pairs of sprouts (that were visible on the 2D projection) uniformly sampled on the surface of a microcarrier. It demonstrates a monotonic relation between 2D and 3D distances.

In order to illustrate potential positive and negative feedbacks of the sprouting process, experimental histograms of all  $d_{ij}$  were compared with a theoretical case with no feedback (Fig. 2d). In this theoretical case, all sprouts were independent and equally likely to form anywhere on the spherical microcarrier. It can be seen that the relative frequencies of the lowest distances (<  $31\mu m$ ) were significantly reduced in the control compared to simulation, but were increased with the administration of DAPT. Compared to the theoretical simulation with no feedback, DAPT appears to restore the sprouting frequency fully in the  $15\mu m$  and  $31\mu m$ , but its effect is moderate at the <  $15\mu m$  distance. The inability of DAPT to restore the frequency at <  $15\mu m$  was likely due to Notch-independent effects of cell and nutrient depletion at this length scale of <  $15\mu m$ . The experimental frequencies at mid range from  $75\mu m$  to  $150\mu m$  were higher than the theoretical, consistent with length scales of paracrine signaling that enhances sprouting.

#### Analysis of Tip Cell Speed

To characterize the effects of Notch signaling on the sprout growth rate, the average speed of tip cells were measured over 48 hours through time lapse imaging in 3D (Fig. 3). The speed of a tip cell was measured once a sprout was formed (Fig. 4a). The average tip cell speed in the control condition was  $6.4\mu m/hr$ . Treatment with DAPT decreased the tip cell speed slightly to  $4.9\mu m/hr$  (p < 0.05 compared to control). Treatment with anti-Dll4 yielded no statistically significant difference from the control. The additions of Dll4 and Jag1 increased the average tip cell speed to  $9.9\mu m/hr$  and  $8.0\mu m/hr$  respectively (p < 0.05 compared to control).

#### **Temporal Analysis of Sprout Formation**

To determine if Notch signaling influenced the synchronization of endothelial sprout formation, time lapsed microscopy movies were analyzed, and sprouts were automatically detected using an algorithm (Fig. 3). To test the hypothesis that there was temporal

synchronization among endothelial sprouts within the same microcarrier, the initiation times of sprout formation were recorded.  $t_{ij}$ , defined as the time of initiation for the *i*<sup>th</sup> sprout on the *j*<sup>th</sup> microcarrier, was recorded. Temporal synchronization can be characterized as the similarity of  $t_{ij}$  within the same *j*. This similarity can be quantified as the intraclass correlation coefficient  $\rho$  of  $t_{ij}$ .

The observation of these initiation times could be modeled as:

 $t_{ij} = \mu + c_j + e_{ij}$ 

where  $\mu$  is the population mean of all observations,  $c_j$  is the microcarrier effect identically distributed with mean 0 and variance  $\sigma_c^2$ , and  $e_{ij}$  is the residual error identically distributed with mean 0 and variance  $\sigma_e^2$ . The variance of the  $t_{ij}$  is then given by  $\sigma_t^2 = \sigma_c^2 + \sigma_e^2$ .  $\rho$  is defined

as  $\frac{\sigma_c^2}{\sigma_c^2 + \sigma_c^2}$ , or in other words, the proportion of variation explained by belonging to the same class (microcarrier). Hence, it takes value between 0 and 1, with 0 being no intraclass correlation (every sample is basically identically distributed and uncorrelated), and 1 being perfect correlation within class (every sample in the same class has the same value).

The estimates of  $\rho$ , given by  $r_a$ , were computed with analysis of variance (Table 1). It could be seen that  $r_a = 32\%$  for the control condition, indicating a moderate level of correlation among sprouts.  $r_a$  was reduced to 11.6% and 14.3% when Notch was inhibited by DAPT and anti-Dll4, respectively (Fig. 4b). In contrast, enhancing Notch signaling via cell treatment with Dll4 and Jag1 ligands led to an increase of  $r_a$  to 37% and 53% respectively.

One source of the differences in intraclass correlation could be due to shifts in the initiation times of the entire population. For example, drug treatments could simply prevent sprouting for some time, causing the intraclass correlation to be artificially increased or decreased because all sprouting events would occur over a shorter or longer time period. To eliminate this possibility, the population statistics of all the sprouts in each condition were plotted (Fig. 4c). It was observed that the average initiation times were the same for all conditions; thus, pharmacological treatments did not skew the sprout initiation times as a population.

#### Discussion

A fundamental understanding of the angiogenesis process requires elucidation of both the temporal and spatial characteristics of sprouting events. Temporal profiles of expression levels for angiogenic molecules, for example, have been shown to be key in the regulation of vascular development [21–23]. Temporal coordination in the initiation of sprouts may be important in determining subsequent angiogenic processes, such as tube formation, vessel fusion, and maturation. By growing endothelial cells on spherical microcarriers and analyzing sprouting with time lapse microscopy and a statistical model, key temporal characteristics were inferred from data sets that were seemingly random and highly variant. This methodology allowed the measurement and analysis of the dynamics of sprout initiation.

As expected from the previously discribed lateral inhibition behaviour of Notch signaling [2–4, 20], inhibition of Notch using DAPT and anti-Dll4 Ab increased sprouting. The Notch ligands Dll4 and Jag1 have been shown to functionally activate Notch signaling in a 3D environment [20, 24–27]. In this study, these ligands inhibited endothelial sprouting, as consistent with past studies [20, 26, 28]. It is interesting that endogenous Jag1 and Dll4 expressions have opposing effects in retinal angiogenesis [29] in that Jag1 expression is positively correlated with angiogenesis. The current study, however, found Jag1 to have

similar anti-angiogenic effects as Dll4 in the *in vitro* sprouting assay. Other groups administering exogenous Jag1 also had similar findings [2, 10, 20]. It is possible that endogenous expression of Jag1 enables an endothelial cell to adopt the tip cell phenotype [30] via autocrine signaling or cis-inhibition [31,32]. On the other hand, extrinsic Notch activation with Jag1 activates Notch for the entire cell population.

The experimental distribution of the number of sprouts for each microcarrier was also consistent with Notch lateral inhibition. Inactivation of Notch relieves lateral inhibition, and causes some of those microcarriers that would otherwise have one sprout to instead have two or three sprouts. This lateral inhibition occurs at the length scale of  $< 31\mu m$ . Apparent promotion of sprouting in some distances at the mid length scales (75 – 150 $\mu m$ ) is consistent with paracrine signaling in angiogenesis [33–35]. It is probable that endothelial cells secrete cytokines such as VEGF to promote sprouting in neighboring cells at an appropriate distance to allow the two sprouts to eventually merge and form a functional new capillary [36–38].

The tracking of average tip cells in this study showed an interesting phenomenon regarding cell migration speed. After endothelial cells are activated to form tip cells, the tip cell speed increased with Notch activation and decreased with Notch inhibition. These results may reconcile the findings of several groups who concluded Notch inhibition decreased angiogenesis [39–41]. In those studies, the *in vitro* assay used were either the 2D morphogenesis assay or the aortic ring assay. It is possible that the activity observed in those assays was predominantly cell migration, which was enhanced with Notch signaling, rather than endothelial activation.

By utilizing the intraclass correlation model, endothelial sprouting was shown to be intrinsically synchronized. This temporal synchronization is inhibited with the inactivation of Notch, and enhanced with the activation of Notch. Previous studies have shown that Notch signaling is critical in creating organized and efficient vasculature, despite reduced vessel density [2–4]. Our study suggests yet another mechanism by which Notch may mediate efficient vascular growth. Presumably, functional vasculature requires fusion of different endothelial sprouts. The temporal synchronization of endothelial sprouting may be critical in coordinating angiogenesis in a neighbourhood of endothelial cells. Past studies have provided great insight about the synchronization of calcium spikes in endothelial cells, suggesting oscillatory release of endothelial-derived substances [42–45]. However, these works had focused on membrane potential oscillations that are on the higher frequencies (e.g. 5–10 min). Furthermore, their effects on sprouting angiogenesis had not been directly investigated. This work is the first to study synchronization in the context of sprouting behavior.

Across conditions, both the estimated population means and variances of the sprout initiation times did not show any difference. Such pattern was unlikely to alter with an increase in population sample size. It is likely that the temporal dynamics in this *in vitro* process were more subtle than could be discerned by simple mean-variance analyses on the population level. This is an equivalent problem to analyzing family data. The numbers of siblings in each family are usually low - the average American family size was 3.19 from 2005–2009 (US Census Bureau). On the other hand, variances of attributes in the population were high - the Gini index of US income is 46.9 in 2006 (US Census Bureau). However, statistical inferences could still be made by sampling a large number of households. Similarly, by taking advantage of the high number of microcarriers in each experiment in this study, the intraclass correlation model compensated for the low sprouting ratio in each microcarrier and the seemingly indistinguishable and noisy population data. This study illustrated that this methodology could be applied in the context of angiogenesis to characterize the otherwise elusive temporal coordination involved in endothelial sprouting.

In sum, this work was the first to address and quantify the temporal synchronization of endothelial sprouts to date. These techniques allow inference of subtle information that would otherwise be drowned in low sample sizes or noisy population data. It is important to point out that this study started with a clear objective of elucidating temporal synchronization, and a specific statistical model is calibrated against the underlying assumptions. Thus, in this setting, a large number of sample size increases the power of the statistical tests. The algorithm and statistical approach developed here could be extended to analyze other cellular events that involve synchronization. This approach can also be automated for large-scale and target-based screens for therapeutic discoveries.

#### **Materials and Methods**

#### Immunohistochemistry of Notch signaling

HUVECS (Human Umbilical Vein Endothelial Cells) were seeded onto coverslips at 37°*C*. For the immobilized ligand condition, previously, cells were starved, and cover-slips were submerged in poly-l-lysine for an hour, with ligand solutions (Jagged and anti-Dll4) were added for atleast 4 hours in RT. The conditions of Control, Starved (for 6 hours) and DAPT in full growth medium were conducted in 24 hours with no previous cell starvation. Samples were fixed with 4% paraformaldehyde for 1 hour at room temperature. Samples were washed two times with PBS, non-specific sites were blocked with Pharmingen Stain Buffer (BSA) in PBS and permeabilized with 0.1% Triton-X+ Normal Goat IgG (1:50) in PBS for 30 min. Samples were immunostained for 45 minutes at room temperature with anti-NICD primary antibody (10ug/ml, 1:100, all antibodies diluted using Pharmingen Stain Buffer). After incubation, samples were washed two times with PBS, once with stain buffer for 5 minutes, and then incubated for atleast 45 minutes at room temperature with secondary antibody, AlexaFluor 647 Goat Anti-Rabbit (Molecular Probes 4mg/ml, 1:500). Finally, samples were washed three times with PBS, and mounted onto slides by using Prolong Gold antifade reagent with DAPI (Molecular Probes).

#### 3D Cell Culture and in vitro Sprouting Assay

Human umbilical cord endothelial cells (HUVECs) were cultured to confluence at 37°C and 5% CO2 in EGM-2MV medium (Lonza) containing all supplements. Angiogenic activity of endothelial cells was assessed using a modification of a widely used in vitro sprouting assay [15]. Briefly, dextran beads microcarriers (Cytodex 3) with a dry weight of 50mg were swollen in PBS and autoclaved. The microcarriers were washed in EGM-2MV medium and seeded with  $3 \times 10^6$  HUVECs in a spinner flask. The microcarriers were stirred for 2 minutes out of 30 minutes for 3 hours at 37°C incubation. After 3 hours, the beads and cells mixture were continuously stirred and incubated for an additional 21 hours. The cell-coated beads were then seeded in fibrin gel in a 24-well-plate. The composition of the fibrin gel in each well was 0.682mg fibrinogen (Sigma, T3879), 11.4µg aprotinin (Sigma, A4529), 0.455U thrombin (Sigma, T6884) in 393µL of PBS and 57µL of EGM2-MV. Gels were incubated at 37°C for 30 minutes and media of experimental conditions were placed on top of the gel.

Media contained the addition of 50ng/mL VEGF. Recombinant human Jag1-Fc (R&D Systems, 1277-JG-050) and Dll4-Fc (Enzo Life Sciences, ALX-201-386-C010), when used, were  $1\mu g/mL$ . Adopted from methods in [24], Dll4-Fc was clustered with anti-Fc antibody (1:1 ratio) at room temperature for 60 minutes prior to addition to the media. When Notch inhibitors were used, the concentrations were 2.5mM DAPT (Sigma-Aldrich, D5942) and  $5\mu g/mL$  anti-Dll4 (gift from Genentech) respectively. DAPT was dissolved in a minute amount of DMSO. All conditions contained the same amount of DMSO (< 0.01%). Media were changed every 24 hours. In the static analysis of the sprouting ratios, cells were

allowed to sprout from beads into surrounding gel over 4 days. After 4 days, the gels were rinsed with PBS and fixed with 4% paraformaldehyde prior to imaging. Subsequent to fixing, samples were stained with DAPI and visualized at 10X objective magnification with an Olympus IX2 microscope. Sprouts were identified as continuous multi-cellular structures extended from the microcarrier beads with a minimum of two cells in the structure.

#### Simulation of Probability Distribution of Sprouts

A sprout was assumed to form anywhere on the spherical surface with equal probability. The distance between each pair of visible sprouts was measured as the induced arc length in the 2D projection on the XY plane. The resulting probability distribution in this 2D projection, as illustrated in Fig. 2b and 2c, was generated as follows:

Sampling the position of a sprout involves two steps: First, the horizontal angle generated by the sprout in Fig. 2b, namely  $\theta$ , follows a uniform distribution over  $[0, 2\pi)$  by symmetry. The infinitesimal sliced surface  $dS = 2r^2d\theta$  shows the area from which a sprout with a given angle  $d\theta$  can possibly reside. Next, the vertical angle  $\phi$  is sampled according to the density  $\frac{1}{2}\sin\varphi$  over  $[0, \pi]$ . These two steps together determine the position of the sprout on the microcarrier's surface. Furthermore, we resample the empirical distributions obtained from our experimental data to generate the number of sprouts on each microcarrier  $n_s$  (Fig. 2a), as well as the radius of each microcarrier r (see Supporting Information for more detailed explanation). The distances between each i-j pair of visible samples were calculated as  $d_{i,j} = r (\min(|\theta_i - \theta_i|, 2\pi - |\theta_i - \theta_i|))$ .

#### Time Lapse Imaging and Tip Cell Tracking

Prior to seeding into fibrin gels, cells were stained with 250ng/mL octadecyl rhodamine B chloride (R18) (Invitrogen O-246) overnight. During the staining process, cells were simultaneously synchronized by serum-starvation. Immediately after seeding into fibrin gels, cells were placed on the microscope stage, time lapse microscopy images were taken for 48 hours. Images were taken with an Olympus IX2 microscope with the CARV II spinning disk confocal imager. The image stacks were taken 60 minutes apart and at  $20\mu m$  Z-resolution. The acquired 4D datasets were analyzed using the spot detection and tracking modules in Imaris detection to track tip cell movements.

#### Intraclass Correlation Model

To analyze the effects of clustering within sprouts in microcarriers, the intraclass correlation coefficient (ICC)  $\rho$  is defined as  $\sigma_c^2/(\sigma_c^2 + \sigma_e^2)$ . As demonstrated by Donner [46], there are multiple ways in estimating the parameter  $\rho$ . Since the number of sprouts on each microcarrier was not constant for all sprouts in this case, the modified analysis of variance intraclass correlation,  $r_a$  was appropriate for estimating  $\rho$  (and also because it was demonstrated to perform better than other estimators, such as MLE, when the value of  $\rho$  is small [47]). This estimation utilized the standard analysis of variance (ANOVA) table and defined MSW as the mean square within microcarriers, and MSA as the mean square among microcarriers. A further parameter  $n_0 = n - \sum_i (n_i - n)^2 (k - 1)N$  was needed to adjust for the non-uniformity of the number of sprouts in different microcarriers, with  $n_i$  being the number of sprouts in the *i*<sup>th</sup> microcarrier, i = 1, 2, ..., k, and  $n - \sum_i n_i/k = N/k$  the average family size. The mean squares within microcarriers and among microcarriers were then  $S_W^2 = MSW$ , and  $S_A^2 = (MSA - MSW)/n_0$  respectively. The modified ANOVA ICC was then:

$$r_{a}{=}\frac{S_{A}^{2}}{S_{A}^{2}{+}S_{W}^{2}}$$

where microcarriers with less than 2 sprouts were not included. The reason was that such inclusions tend to increase the standard error for  $r_a$  when both  $r_a$  and the number of sprouts per microcarrier was small [17]. The significance of  $r_a$  could be evaluated with an F-test. These statistical inferences were derived in the supporting information.

The estimation of  $\rho$  using analysis of variance made several assumptions: 1)  $c_j$  and  $e_{ij}$  are normally distributed, 2) samples across classes are independent, and 3) homoscedasticity across classes. As derived and discussed in [48–51], the estimate of ICC is robust against the normality assumption. Independent sampling was assumed from the experimental settings since microcarriers were far apart from each other (> 500µm). Homoscedasticity across classes was confirmed using Bartlett's test and Levene's test, respectively with p-values of 0.596 and 0.879, indicating that the null hypothesis of equal variance cannot be rejected.

#### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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(a) Immunostaining with Notch ligand treatments



(b) Representative images of sprouting ratios

(c) Quantification of sprouting ratios

#### Figure 1.

*In vitro* endothelial sprouting in response to Notch ligands and inhibitors. (a) Representative images of cell-seeded dextran beads embedded in fibrin gel under different conditions. Images are compositions of Z-stacks of 50 Z-planes at 3  $\mu m$  apart. A sprout was defined as a multicellular extension with >1 connected ECs that were attached to the microcarrier (see arrows). (b) Immunostained images where cells were stained for activated Notch-intracellular domain (NICD) and with DAPI. "Full" refers to the fully supplemented media and "starvation" refers to serum starvation for 6 hours. The scale bar represents  $100\mu m$ . (c) Quantification of the number of sprouts per bead at different conditions. Sprouting ratio refers to the treatment with both Dll4 and anti-Dll4 antibody. Values represent means and error bars represent standard errors (n - 7). One and two stars represent, respectively, p < 0.05 and p < 0.01 statistical significance from control with Bonferroni correction.



(a) Distributions of the number of sprouts per bead



(b) Spherical Coordinates



(c) 2D maximum intensity projection of sprouts on a microcarrier bead



(d) Distribution of the distance between each pair of sprouts

#### Figure 2.

Analysis of sprout spatial distribution. (a) Histogram of the number of sprouts on each microcarrier bead (number of microcarriers >1200). (b) Coordinate system used to define distances measured, and to generate the theoretical histogram if all sprouts are uniformly distributed. (c) Composite images of three dimensional stacks at 48h. The reconstruction showed the representative image of a microcarrier with 7 sprouts (indicated by green arrows). The distance between each closest pair of sprouts was measured and indicated by yellow arcs with double ended arrows. (d) Histogram of the distances between each pair of sprouts for control (red) and DAPT treatment (blue) (n>1000 each condition). The

theoretical distances if all sprouts were equally likely to appear at each spot on the microcarrier was illustrated in green.





#### Figure 3.

Automated sprout tracking system with sample fluorescent images at 9 different Z-slices. Cyan circle represents the location of the microcarrier detected by maximization of the accumulation function in the Hough transform. Yellow circle dash-dotted circle represents the threshold a cell protrusion had to cross to be counted as a sprout. Blue dashed circle represents the outer limit, outside of which no sprout is counted. Green star represents a sprout present at the current z-slice. In the image of z = 4, sprouts from all planes are indicated, denoted by a cyan number representing the z-slice that the sprout is present in.





#### Figure 4.

Temporal aspects of sprouting. (a) Average speed of the tip cell at each condition (*n* 135 for each, where each sample is the average speed of one tip cell). Bars represent means and error bars represent standard errors. Statistical significance was computed using multiple comparison ANOVA with Tukey's honestly significant difference criterion (\* represents *p* < 0.05). (b) Intraclass correlation coefficients for different conditions. Errors bars represent standard errors estimated. \* and \*\* represent, respectively, *p* < 0.05 and *p* < 0.01 significance levels compared to control as tested using the two-sample *Z*-tests in [52]. (c) Population statistics of the means and standard deviations of sprout initiation times.

# Table 1

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	Control	DAPT	anti-Dll4	DII4	Jag1
F-statistic	3.0786	1.6081	2.7562	7.7186	16.2438
$r_a$	0.321	0.1157	0.1427	0.3665	0.5257
$p$ (null of $r_a = 0$ )	0.0000	0.0137	0.0000	0.0000	0.0000
N (# of sprouts)	360	236	1510	945	699
k (# of microcarriers)	170	69	312	450	381
μ	17.97	17.50	18.38	18.43	17.26
Standard error	0.00339	0.0037	5.73E-04	0.0019	0.0032