Quantitative analysis of traction forces exerted by translocating neuronal growth cone

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Abstract

This study involves traction force microscopy related techniques for analyzing the process of neuronal growth cone dynamics in the context of axon guidance. The system that is used will allow measuring the forces exerted by filopodia as they translocate on a flexible gel surface. This gel has an array of fluorescent fiduciary marker beads, which get displaced along with the deformation of the gel. Vector maps for gel deformation can be constructed by using bead tracking algorithms for image analysis. These vector maps would allow estimation of the forces exerted by various parts of the growth cone during the process of axon guidance to get an idea about the dynamics of filopodia mediated motility.

Introduction

Cells interact with their physical environment in various ways. These interactions depend on both, the biochemical status of the cell as well as mechanical characteristics of the environment. Recent studies have focused on the importance of stiffness of the substrate on factors like cell motility, morphology and survival (1). Cell motility is achieved by applying traction forces on the substrate. These forces, exerted in different parts of the cell, are controlled in a coordinated way (2). This coordination is brought about by complex signaling mechanism which is not clearly understood.

Morphology and dynamics of a neuronal growth cone tend to be modulated severely by the physical characteristics of the environment. A neuronal growth cone uses multitude of cues from its local environment and carries out the process of axon guidance. A growth cone needs to differentiate between identity and magnitude of guidance cues on its small spatial scale. This process requires a temporally coordinated effort for sensing its environment and then responding to it accordingly.

Cells have specialized transmembrane receptor proteins called integrins which bind to Extra-Cellular Matrix (ECM) proteins and induce an attachment between cell and the substrate. Integrins interact with proteins like Talin which in turn interact with actin filaments (3). These proteins interact with other associated proteins and form protein complexes at various foci along cell-substrate interface. These protein complexes are known as focal adhesion complexes and they have a central role in mediating the mechanical signaling between cell and substrate. The interaction between acto-myosin cortex and focal adhesions enable a cell to produce protrusive forces with help of specialized protrusive structures like, lamellipodia and filopodia. These structures are enriched in nascent focal adhesion. Focal adhesions undergo constant turnover; nascent focal adhesion undergoing maturation and then degradation after some time. It is suggested that the forces exerted by these focal adhesions vary with time as they mature (4). A cell has an ensemble of these focal adhesions which exert forces in a spatially coordinated way. A complex signaling circuit brings about coordination across different parts

of the cell; inducing directional cell motility. It is not clear whether this signaling is chemical or mechanical and suggestions are that it is a combination of both.

A growth cone is guided along a defined path by coordination of the traction forces in different parts of the growth cone. Some studies have pointed out that different proteins, involved in the integrin mediated signaling pathways, are regulated differently even in the local environment of a growth cone (5). The growth cone has focal adhesions with varied molecular composition and this variation might correlate with the positive as well as negative signals that guide a growth cone (Ghose A., unpublished data). This, combined with the turnover of focal adhesions, makes it interesting to investigate the correlation between variation in the composition of focal contacts and the forces exerted by them. A clear picture of the whole process can be obtained by performing quantitative measurements, like generating a force map using traction force microscopy.

Traction force microscopy has been used to study lamellipodia mediated motility in fibroblasts (6). A neuronal growth cone, on the other hand, is a very different structure in many ways. A lamellipodium has smooth boundary whereas a filopodia has a characteristic elongated projections. The primary Rho GTPase involved in producing the two types of structure is also different (7). A growth cone possesses a passive axonal tail which affects the growth cone dynamics as well. Hence, growth cone dynamics can not be understood by extrapolation of lamellipodial dynamics.

A study has looked at few aspects of filopodia mediated motility in neuronal growth cones at scale of a single filopodia (8). Current study aims to produce traction force map for an entire growth cone. This force map can give information about dynamics of a growth cone at global scale. Current experimental setup has a few novelties of its own. The substrate preparation methods are highly robust, reproducible and allow us to carry out experiments in bulk.

Materials and Methods

Primary neuronal cultures

The chicken embryos were incubated in an intact egg shell at 37^{o}C in a humidified incubator. The embryos were dissected at various stages depending upon the source tissue. Dorsal Root Ganglion neurons were dissected out on 8-10 day of incubation, whereas the Spinal neurons were dissected out on 4-5 day of incubation.

The dissected tissue was temporarily stored it in Embryo Medium (Dulbecco's Modified Eagle Medium (DMEM D6546 SIGMA, St. Louis, MO) / Leibovitz Medium (L15 12-700F LONZA, Walkersvilla, MD) + 2mM L-Glutamine + A5955). All explants were transferred to Dissociation Medium as soon as the dissections were done. Dissociation medium has components like Trypsin (T4549 SIGMA) which enzymatically degrades cell-cell contacts. The explants were transferred to Trituration Medium (Hank’s Balanced salt Solution (HBSS 55037C SAFC BIOSCIENCES, Lenexa, Kansas) + A5955). While cell were in this medium, shearing forces were applied to physically dissociate the cells from each other. The cell mass was then mixed with Base Culture Medium (DMEM / L15 + 2mM L-Glutamine + A5955 + 10% FBS) and plated on top of the gel surface.

Activation of glass surface

Surface of a cover glass was treated so that polyacrylamide gels could adhere to it. The glass surface was treated with 1N HCl for 2-3 hours at 60^{o}C to remove any dirt or grease from the glass surface. The glass surface was then treated with 0.5% solution of 3-aminopropyl-trimethoxysilane (281778 SIGMA-ALDRICH, St. Louis, MO), diluted in WATER, for 10 min. The Silane solution was thoroughly washed off from the glass surface using multiple WATER washes. The cover glasses were left at 160^{o}C for 1hour. The cover glasses was treated with 0.5% solution of Glutaraldehyde (G5882 SIGMA-ALDRICH), diluted in PBS, for 30min, after the glass surface had cool down. The glass surface was thoroughly washed and was allowed it to dry vertically.

Preparation of Polyacrylamide gels

The required volume of the gel was calculated by considering parameters like, thickness and surface area of the gel, number of gels, etc. Volume of the gel mix was taken to be at least 5 times that of the required volume to ensure that gels prepared in one batch were similar to each other.

Necessary volume of 30% acrylamide mix (29% acrylamide + 1% bis-acrylamide A3574 SIGMA-ALDRICH) was taken and sonicated fluorescent beads (FluoroSpheres 505/515 F8811 INVITROGEN, Carlsbad, CA) (at 1:1000) were added to this mixture. Required amount of water was then added to make volume. 10% Ammonium Persulfate (APS A3678 SIGMA) (at 1:100) and Tetramethylethylenediamine (TEMED T9281 SIGMA) (at 1:3000) was added to initiate the polymerization. The mixture was immediately poured onto an acid washed cover glass. This surface was overlaid with Glutaraldehyde treated cover glass to make a sandwich. The fluorescent beads start to settle on the bottom of the sandwich as the gel is polymerizing slowly. Clean cover glass was peeled off after the gel had polymerized.

Functionalization of gel surface

ECM proteins are conjugated with gel surface so that the surface becomes suitable for cell culture. The protocol can be used for various ECM proteins like, Collagen, Fibronectin, Laminin, poly-L-lysine, etc.

Small volume of Hydrazine Hydrate was poured on top of gel and the gels were incubated for 4 hours at room temperature. The gels were washed with 5% Acetic acid for 1 hour followed by 1 hour and washed wash with water. The oxidization reaction of ECM proteins was setup by mixing stock solutions; 2x ECM stock prepared in 0.1 N Acetic Acid; 4x Acetic Acid stock; 10x Sodium Acetate stock and 10x Sodium Periodate stock. The reaction was done for 30 min at pH 4.1. The gels were coated with oxidized ECM for 1 hour. The gels were washed 2-3 times with PBS. They were either stored in Phosphate Buffered Saline at 4^{o}C or were kept in the incubator dipped in Base Culture Medium to allow equilibration and were used for cell culture.

Stiffness measurement of Polyacrylamide gel

1 gel sample from a batch 6 was used for Atomic Force Microscopy (AFM) analysis so that the stiffness of the gels could be determined. In principle, the process need not be repeated for each batch and a standardized protocol can be created so that there will be a correlation between substrate stiffness and substrate composition.

An AFM cantilever of stiffness value similar to that of the sample was used to probe the surface of the gel. The cantilever was calibrated with respect to the cover glass base. Forces were applied on the surface of the sample to record the displacement that is caused by the response of the sample in an action-reaction scenario. A force curve was plotted using the data generated in this experiment. The linear region of the plot was selected for calculating the stiffness of the sample. The AFM (JPK instruments NanoWizard 2) assumed a Hertzian model to fit the force curve and it calculated the Young's modulus. The sample was probed at more than 20 randomly chosen locations for calculating the averaged stiffness.

Imaging and analysis for measurement of traction forces

Images were recorded using fluorescence microscopy. Fluorescent beads were tracked to estimate the deformations in gel. A time lapse imaging was carried out to get a sequence of images from which the displacement of beads could be measured.

The image processing carried out using an open source algorithm written in MATLAB (The MathWorks, Natick, MA). The algorithm thresholds the images to isolate bright areas corresponding to beads. Then the algorithm identifies the bright spots of a given size as beads. This given size corresponds to the diameter of a bead in terms of its pixel size. The diameter needs to be manually provided as an external parameter and needs to be determined manually. Different set of images might need different threshold values which should be determined manually.

The algorithm compared the bead position across different images in the image sequence. For this purpose it used specified value for a parameter called 'maximum displacement'. The parameter

dictates the limit to which a particle can travel across two frames. The value for ‘maximum displacement’ should be less than average inter-particle separation. Ideally, the imaging should be done in such a way that the bead displacement across two frames would be comparable with its diameter. A suitable value for ‘maximum displacement’ was inferred from analyzing the distribution for values of velocities for all particles. The value of ‘maximum displacement’ for which the particle velocity distribution showed lowest standard deviation was chosen for data analysis.

With respect to a particular particle at time point ‘t’, algorithm identified various particles within a distance given by maximum displacement in the image at time ‘t + 1’. The particles in the two images were combinatorially correlated and sum of their ‘square of displacements’ were calculated. The combination for which the sum of displacements turned out to be minimum was considered to be the correct correlation. Iterating this process for various consecutive frame combinations generated particle specific tracks. These tracks give information about the displacement of the beads and can be combined with stiffness data to yield force map.

Results

Traction force microscopy is a technique developed for analyzing cellular dynamics by measuring the forces exerted by a cell on its surrounding substrate. It combines cell culture techniques with microscopy and image analysis to produce a force map which depicts the distribution of forces exerted by a cell on its surroundings. Cells are grown on transparent flexible substrate which has fiduciary markers embedded in it. The substrate deforms due to forces that are applied by the cell. This deformation is measured by analyzing a series of images to generate a displacement map. The data is then combined with stiffness measurements of the substrate to produce a force map.

A variation of traction force microscopy has been developed in this experiment. The protocols described here are formed by studying those described in (9, 10, 11). One continuous procedure was compiled by doing an appropriate mix and match of different parts of these protocols and they were re-optimized for compatibility. The final protocol was standardized in such a way that it will be compatible with experiments involving forward and reverse genetic approaches.

Activation of glass surface and polyacrylamide gel preparation

Optically transparent and substantially adherent glass surfaces were produced so that polyacrylamide gels can be cast on. During the experiments it was concluded that the Glutaraldehyde treatment is a crucial step that needs optimization. Optimum time for the Glutaraldehyde treatment turned out to be 30 minutes. A treatment for 20 minutes produces insufficiently adherent surface, whereas a treatment for 40 minutes produced highly adherent surface although the surface had red opaque spots. Fig 1A shows a cover glass that was treated for 30 minutes and Figure 1B shows a cover glass treated for 40 minutes, which had the opaque red spots. Figure 1C and D show a comparison between stability of the gel on treated and untreated glass surface. The procedure is best suited for gels that have a lower concentration of acrylamide mix (less than 15%). The gels became progressively less adherent as the concentration of acrylamide mix increased (data not shown). Gels of uniform thickness were produced by sandwiching them between a Glutaraldehyde treated cover glass and an acid washed

cover glass (Figure 1E). Gel adhered to the Glutaraldehyde treated cover glass. Acid washed cover glass was peeled off once the gel had polymerized.

Fiduciary fluorescent beads were added to the gel. These beads were made to settle very close to the top surface of gel. Figure 2A and B show z-sections that are 10 µm apart; focal plane in 'A' being same as the surface of the gel and that in 'B' is inside the gel. Polymerization was slowed down by reducing the concentration of TEMED so that beads will have sufficient time for settling down. The gel was cast on top of clean cover glass and it was overlaid by the activated cover glass with activated surface in contact with gel. This modification produced desired result with less complication as compared to other suggested methods.

Conjugation of ECM proteins on polyacrylamide gel surface

Stock solutions were prepared as mentioned earlier. Hydrazine Hydrate was used to conjugate oxidized Fibronectin (F4759 SIGMA), at a concentration of 1 mg/ml, to the polyacrylamide surface. Hydrazine Hydrate reacts with polyacrylamide surface and forms a covalent bond. The other domain of Hydrazine Hydrate reacts with the oxidized R group of Lysine in Fibronectin. Hence the Fibronectin gets covalently linked to the polyacrylamide surface. Cells don't attach to gel surface if it is not treated with Fibronectin, hence Fibronectin conjugation is inferred from cell attachment. A quantitative assay was not necessary because Fibronectin was in excess.

Primary neuronal cultures on gel surface

Neurons were grown on top of protein derivatized polyacrylamide gels. Figure 3A and B show representative images of neurons grown on gel surface and glass surface. The cells were grown on glass surface to have a positive control for checking cell survival. The gel-coverslips were currently placed in 6-well plates. Cells tend to get washed off from the gel surface due to local currents in the cell culture medium, while the plate is being moved from one place to another. The difficulty was overcome by reducing the amount of medium per well to 700-800 µl, but gels can desiccation due lack of medium if sufficient care is not taken.

Image analysis

Images were processed manually to determine parameters like, diameter of a bead in terms of pixels (should be an odd number), fraction of maximum intensity that can be set as a threshold for filtering out noise. Figure 4A shows an image containing bead along with surrounding area which contains noise. Figure 4B has the intensity distribution for same image. Figure 4C shows same image as 4A but the area under consideration corresponds only to noisy region. Figure 4D shows the intensity distribution for the area selected in 4C. We can estimate the threshold by looking at intensity distributions in 4B and 4D. Diameter of the bead, in terms of pixels, can also be determined using image of the bead in 4A or 4C.

Another parameter, maximum displacement, needs to be determined using trial and error while optimizing for velocity distribution that has least standard deviation. Figure 5 depicts the optimization process. The image analysis code is run, for a set of test images, at different values of maximum displacement. Figure 5A-E show velocity distributions for these runs (threshold = 0.5; diameter of the bead = 7). The value for maximum displacement that gives least variation in velocity is chosen to be 6. Figure 5F shows initial position of the particles (red spots), final position of particles (green spots) and algorithm generated particle specific tracks (blue streaks). The data would be used to generate force map.

Gel stiffness measurement using AFM

Gel stiffness was measured in terms of its Young's Modulus using an AFM. These measurements of the Young's Modulus are at microscopic scale, whereas an estimate at the spatial scale of a growth cone would be more relevant. Stiffness measurement at macroscopic scale can be done using specialized round tip cantilever of AFM.

Data for the measurements at microscopic scale is presented here. The values of Young's Modulus vary a lot within a single sample itself, hence reproducibility of this data can not be guaranteed. Measurements in current setup are not reproducible. Literature suggests that the variation in

stiffness is less in case of stiffer gels hence the analysis was carried out on 30% gels. The gel is probed at various places in a sample and local stiffness values are averaged to get the global stiffness. A schematic for this experiment is shown in Figure 6. The randomly distributed red spots would indicate places where the stiffness measurement is carried out. The stiffness values are then averaged to get an estimate of stiffness of the gel in terms of its Young's Modulus. Young's Modulus values in two separate experiments are 392.7 kPa (SEM 195.2 kPa, n = 41) and 119.2 kPa (SEM 11.2 kPa, n = 53) for 30% gels.

Discussion

The substrate needs to be optically transparent so that microscopic techniques can be used. It can loose its transparency if activation of glass surface and polyacrylamide gel preparation is not observed carefully. Use of polyacrylamide gel as the flexible substrate has advantages of its own. There are other alternatives for polyacrylamide like, PDMS, Silicone rubber, Agarose gel, etc but all of them have some drawbacks. E.g. Silicone rubber can not produce a large range of stiffness; PDMS gels are not stable over long periods of usage; protein conjugation with Agarose is not straight forward. A polyacrylamide gel is transparent which makes it the most suitable gel for microscopy techniques. Its stiffness can be modified easily by varying the concentration of monomer and cross-linker so as to produce a wide range of stiffness. Its stiffness properties don't change over time if stored in hydrated conditions. It is easy to prepare, inexpensive. It requires minimal chemistry to conjugate proteins with it. It is biologically inert hence the interaction between gel and the cell is mediated via ECM proteins only. The chemicals necessary for casting the gel are readily available. The method of sandwiching the gel improves the optical property by producing gels of uniform thickness.

Sulfo-SANPAH (Sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate) or carbodiimide EDC (l-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl) (along with 2-(N-morpholino)ethanesulfonic acid and N-hydroxysuccinimide) can be used for conjugating ECM proteins with the gel surface, but they have various disadvantages e.g. sulfo-SANPAH suffers from limited shelf life, poor solubility, rapid decrease of cross-linking activity when solubilized and dependence on UV lamp power and positioning; resulting in uneven and inconsistent conjugation. The other alternative carbodiimide ECD produces durable conjugations, but it is hard to carry out the reaction (although it might be suitable for producing conjugations in an automated system). Both of them are not useful for procedures like micro-contact printing, which is a very important technique for producing patterns of ECM protein on a surface. These patterns provide a confined space for cell attachment and can be useful for observing dynamics of cells under spatially constrained conditions e.g. it will be worth looking how

this affects growth cone dynamics when a neuron is forced to turn because of spatial constrains produced by pattern of Fibronectin or say, Laminin. Hydrazine Hydrate in combination with oxidized ECM proteins can be used for producing such patterns of ECM proteins and it can overcome the difficulties imposed by earlier methods.

Few standard techniques that are used for measuring the stiffness of a polyacrylamide gel are rheometry, uniaxial testing, AFM and micro-ball indentation. First two techniques give an idea about the stiffness at macroscopic scale whereas the other two measure the stiffness at microscopic scale. There is a large amount of variation that is observed between the resultant stiffness values for gel sample of same composition when measured using various techniques (12). Each of the techniques have advantages and disadvantages associated with them e.g. uniaxial testing or rheometry are easier to perform, but give a bulk estimate; AFM and micro-ball indentation are suitable for scale at which the forces are exerted, but they are not easy to perform and they need expensive instrumentation. Measurements at microscopic scale are necessary, as far as this experimental setup is concerned. Hence uniaxial testing or rheometry won't be of much use. AFM and micro-ball indentation have their positives and negatives. Micro-ball indentation is a nice technique for measuring the stiffness at the spatial scale of a growth cone, but it is subjected to high level of human error. AFM is highly accurate but the measurements are carried at much smaller scale as compared to the growth cone. Stiffness measurements carried out at this scale also respond to inhomogeneities in the gel which are not important from a cell's perspective, hence this will result into irrelevant data. An AFM cantilever with 10 μm diameter tip can make use of the positives of the two systems and can overcome their drawbacks. Hence using such a cantilever would improve the existing experimental setup and provide a relevant insight about the gel behavior.

The image analysis method used here is capable of producing a force map for a translocating growth cone at every time point. This capability will allow us to analyze the dynamics of a growth cone in a detailed way using which it would also be possible to develop statistical models for growth cone dynamics. Image analysis can also be carried out using methods like 'correlation based optical flow'

(13), although a comparison between that and the image analysis method used here is out of the scope of this article and hence it is not presented.

The overall protocol is developed in such a way that it will be robust and highly reproducible. The experiments can be carried out in bulk, allowing one to combine it with other forward and backward genetics approaches. The quantity of chemicals used is also very low as compared with other descriptions, which makes it highly cost efficient.

Conclusion

This article suggests a modification of traction force microscopy technique. This modification will increase the compatibility between traction force microscopy and other wet lab techniques in biological research. Here, a traction force map is developed as a marker for response of a growth cone towards its environment. The changes in the force map can provide insight about the changes in the dynamics of growth cone due to relevant pharmacological and genetic perturbations. By tagging various proteins that are components of a focal adhesion assembly a correlation can be drown between the traction forces and molecular composition of focal adhesions, which can provide insights about processes involved in maturation of focal adhesions. The study can find its applications in fields like cancer metastasis where cell motility is involved.

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Figure legends

1. ‘A’ depicts cover glass that received a 30 minute Glutaraldehyde treatment whereas B show cover glass which was treated for 40 minutes and consequently, which has red colored opaque spots. C and D compare the adhesion on acid washed and Glutaraldehyde treated cover glass. E shows a gel with uniform thickness which was obtained by sandwiching the gel between an acid treated cover glass and a Glutaraldehyde treated cover glass.
2. Figure 2 shows images of beads embedded in gel. ‘A’ shows the focal plane corresponding to the surface of the gel and a focal plane below the gel surface is shown in B. The focal planes in A and B are 10 μm apart. Both scale bars are 10 μm in length.
3. Spinal neurons growing on Fibronectin are shown in these Figures. ‘A’ shows a neuron on a gel-coverslip. B shows that on a glass-coverslip. Both scale bars are 10 μm in length.
4. Few primary steps in image analysis are depicted in this figure. A and C correspond to same area in an acquired image. The regions in the black boxes are analyzed for plotting histogram of intensity in B and D, respectively. The histogram in B represents area with bead as well as noise whereas that in D represents only noise. Using these values a threshold of 200 (~ 0.8 of maximum intensity) can be estimated.
5. This figure has velocity distributions for various values of ‘maximum displacement’. These values are 3, 4, 5, 6 and 7 for A, B, C, D and E, respectively. Visual estimate suggests that the value of ‘maximum displacement’ can be set to 6. Figure 5F depicts particle specific tracks.
6. The figure show a schematic of the spots at which stiffness measurements are done. An average is taken for the measured stiffness values at these spots to calculate stiffness of the gel.

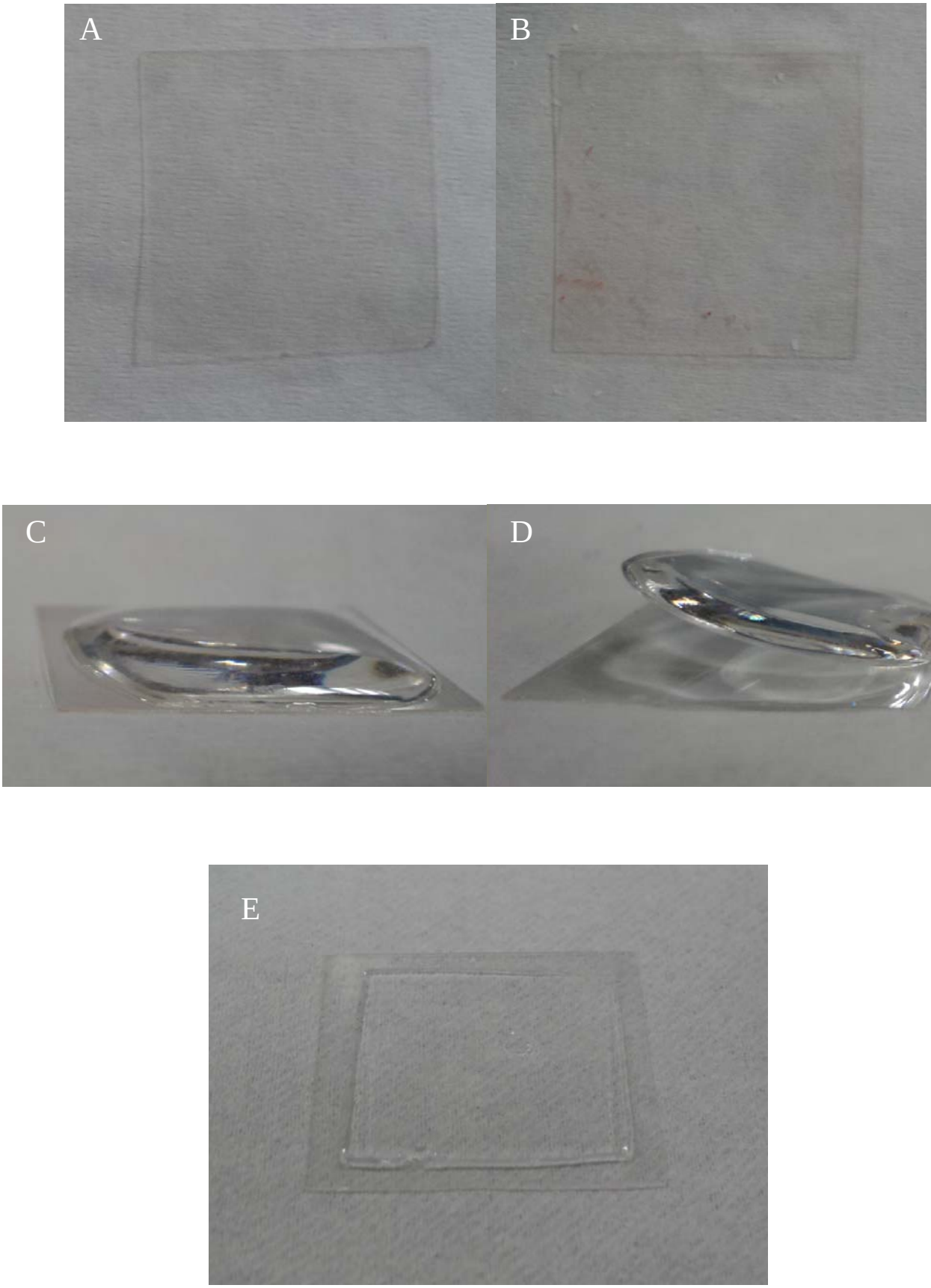
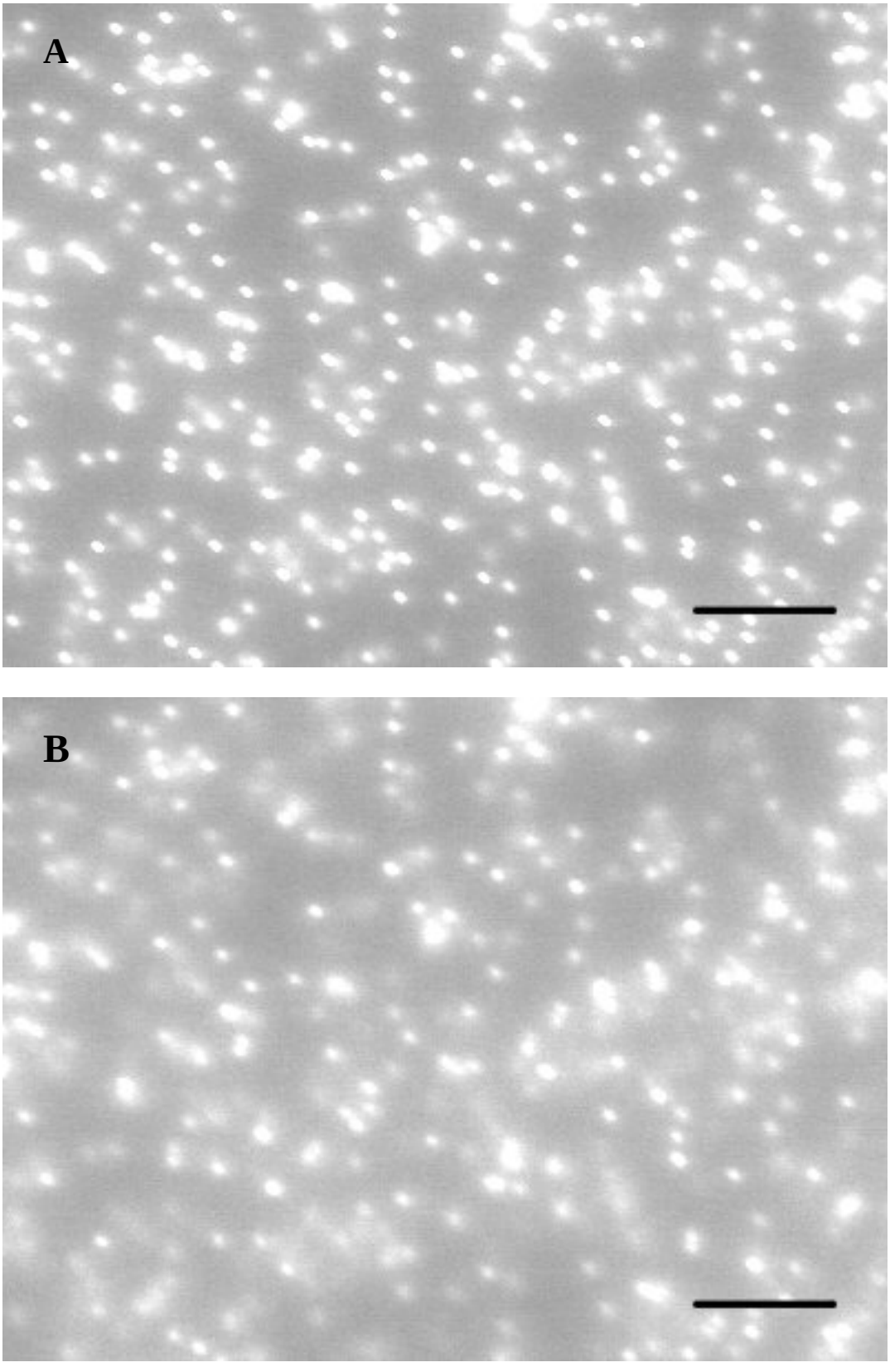


Figure 1



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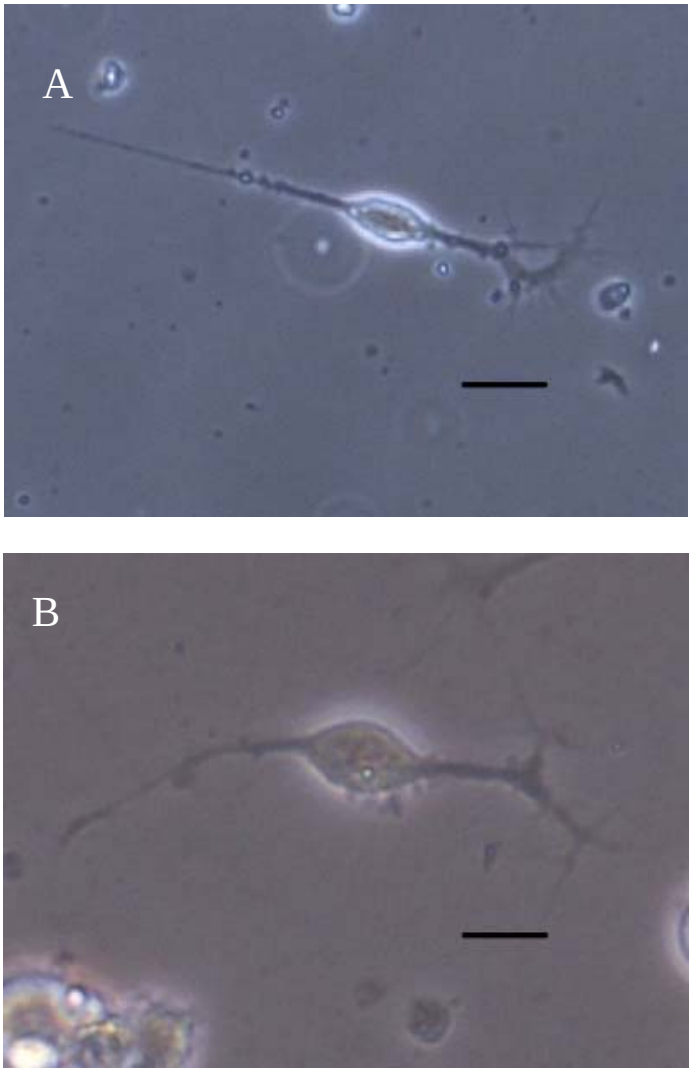

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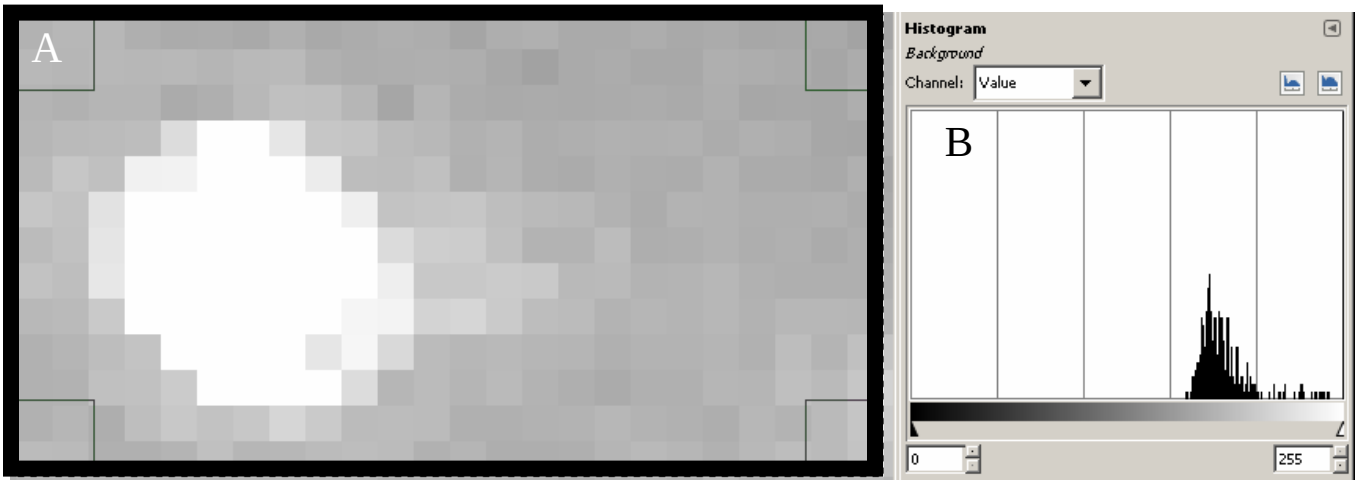
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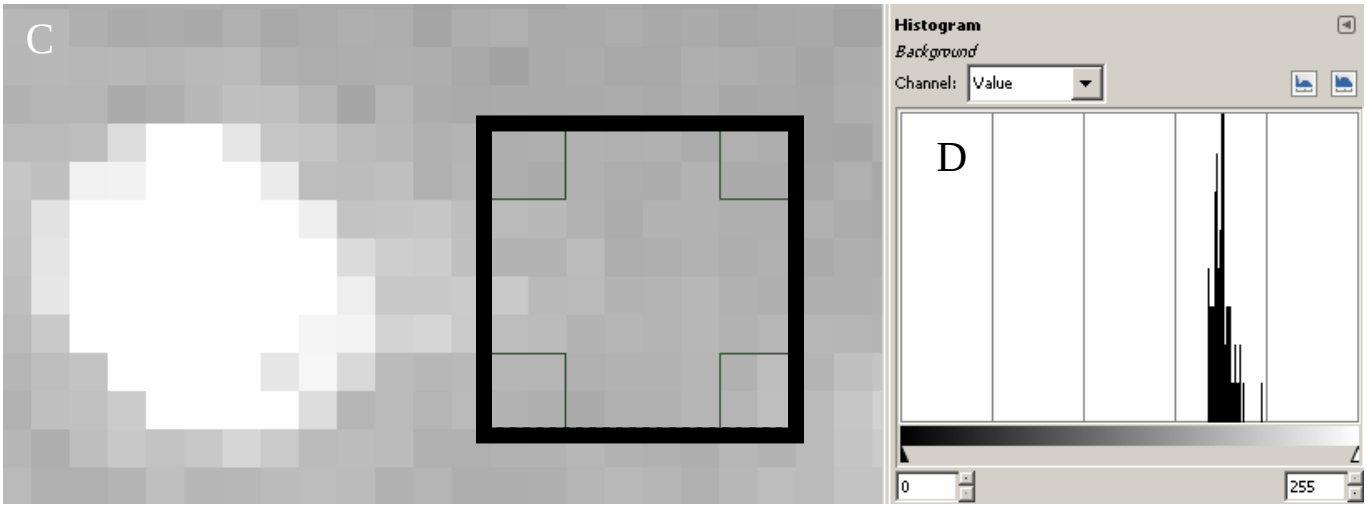
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Figure 4

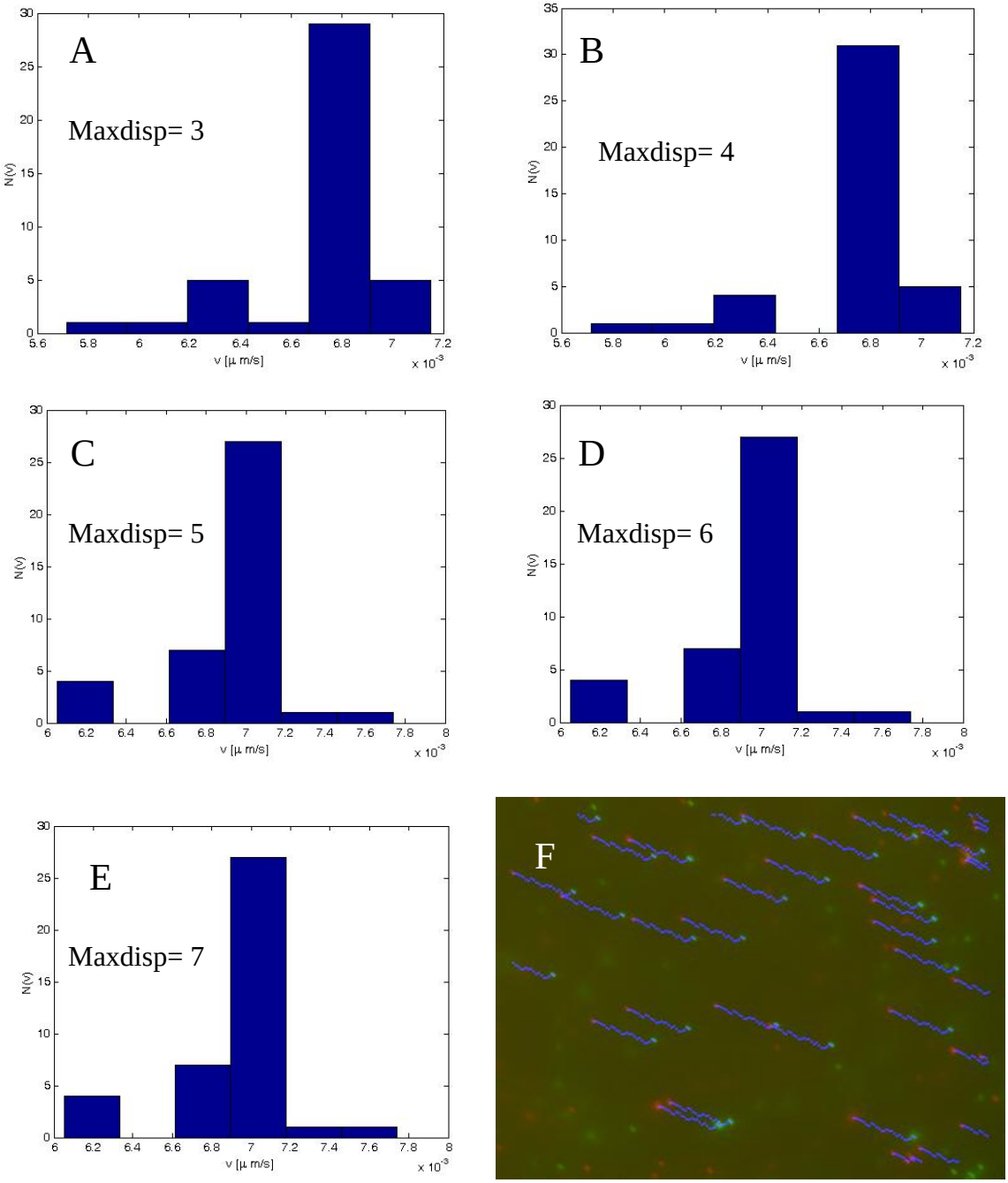
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Figure 5

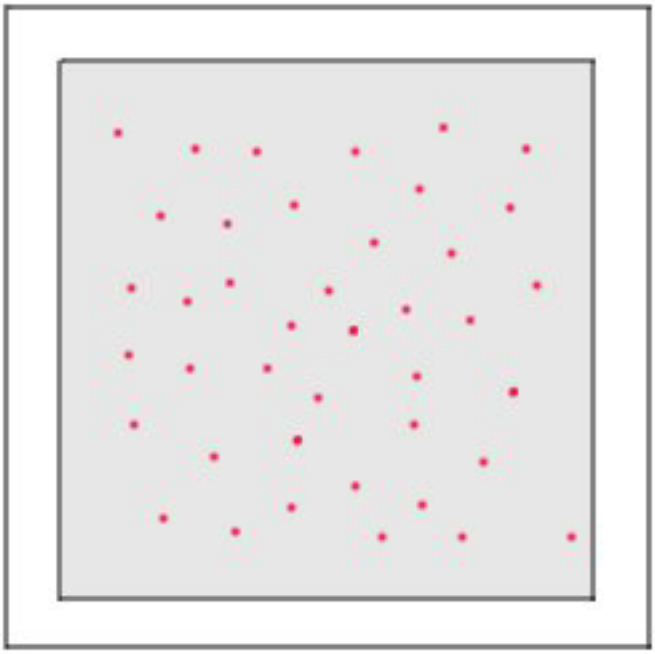

Figure 6