**Determination of the elastic moduli of a single cell cultured on a rigid support by force microscopy**

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**ABSTRACT**

The elastic response of a living cell is affected by its physiological state. This property provides mechanical finger prints of a cell dysfunctionality. The softness (kPa range) and thickness (2-15 µm) of mammalian cells imply that the force exerted by the probe might be affected by the stiffness of the solid support. This observation makes infinite sample thickness models unsuitable to describe quantitatively the forces and deformations on a cell. Here we report a general theory to determine the true Young’s moduli of a single cell from a force-indentation curve. Analytical expressions are deduced for common geometries such as flat punches, paraboloids, cones, needles and nanowires. For a given cell and indentation, the influence of the solid support on the measurements is reduced by using sharp and high aspect ratio tips. The theory is validated by finite element simulations.

**KEYWORDS**

Cell nanomechanics, elastic properties, force spectroscopy, contact mechanics

**INTRODUCTION**

The atomic force microscope (AFM) provides a variety of approaches to determine the mechanical properties and interactions of living cells (1-3). In particular, the elastic moduli of cells have been extensively measured (4-17) because of its relationship to the physiological and pathological state of the cell (1,4,18-20). This observation has led to a variety of AFM measurements to distinguish cells in a variety of diseases (21-30). The implications of force spectroscopy measurements on mechanotransduction processes and their potential for diagnostics have raised a variety of questions. Those concerns range from fundamental aspects such as the contact mechanics model to describe the cell’s deformation (5,31-33), the linearity of the mechanical response (5,7,14,33), the influence of the probe’s dynamics (34) or the interplay between elastic, viscoelastic and energy dissipation processes (35-38). Relevant experimental issues are focused on the determination of the contact point (39), the calibration of the probe’s force constant (40-41) or the influence of the hydrodynamic drag of the cantilever on the force measured by the AFM (42-43).

The most fundamental and long standing issue is the choice of the contact mechanics model needed to transform force-indentation curves into elastic moduli. Contact mechanics models based on Hertz, Boussinesq or Sneddon (44-46) theories are routinely applied in cell nanomechanics (1,4,6-7,9-11,20-24,29,41) . Those models provide analytical expressions that relate the Young’s modulus, indentation and force (45-47). A key assumption in the calculations is to consider the sample with an infinite thickness. Semi-infinite contact mechanics models neglect the influence of the stiffness of the substrate used to culture the cell on the determination of the elastic properties. This assumption might introduce significant errors in the determination of the Young’s modulus of a cell (8). In fact, this concern has been considered in macroscopic measurements performed on thick layers (48) years before the invention of the AFM and its application to cell nanomechanics.

In an AFM experiment, the stress applied by the probe could propagate through the cell to reach the substrate, then reflected back to the cell‘s surface and modify the cantilever’s deflection. This effect could be a major source of quantitative errors in the determination of the Young’s modulus of a cell. There are several factors that make this effect more relevant on living cells than in any other finite soft sample. First, the cell’s thickness is in the 2-10 μm range while a typical AFM experiment generates indentations between 200 nm to 2 μm, this is, about 20% of the cell’s thickness. Second, typical Young moduli values for mammalian cells are in the kPa range, this is, about 8 orders of magnitude smaller than the Young’s modulus of the glass substrate supporting the cells.

Several years ago Dimitriadis *et al.* discussed the influence of the substrate on the cell’s elastic properties as measured by an spherical AFM probe (5). This effect was later described as the bottom effect artefact (8). Dimitriadis theory enables to determine the force exerted by a spherical tip on a cell as a function of the indentation (5). The theory provides an expression to determine the Young modulus without the influence of the solid support.

The bottom effect correction proposed by Dimitriadis *et al.* has three limitations. First, this approach is hard to generalize to other relevant geometries. Second, the method has not been validated by numerical simulations and/or other theories. Third, it treats the variation of the contact area with the indentation as in a Hertzian contact.

Here we present a general theory to determine the elastic moduli in a force-indentation experiment of a thin soft layer, in particular, a living cell attached to a solid support. We show that the forces measured by the AFM on cells are affected by the solid support. This effect is unavoidable in force microscopy. It is a consequence of some cell’s properties such as its finite thickness, softness and incompressibility. We show that the bottom effect artefact is controlled by the ratio between the contact radius and the cell thickness and how this ratio changes with the indentation. The bottom effect elastic theory describes the above features while enabling the determination of the true elastic modulus of the cell without the influence of the solid support.

The theory is valid for any axisymmetric tip shape, in particular spherical, conical, flat punches, needles and nanowires. The theory provides the force as a function of the indentation and contact radius as a sum of terms expressed in powers of the inverse of the sample thickness. We show that those expressions converge when the number of terms used in the approximation is increased. We also show that the theory matches the forces and deformations given by finite element simulations.

**MATERIALS AND METHODS**

**Theory of the forces and deformations of a thin sample on top of rigid support**

Figure 1a illustrates a force microscopy-cell interface. Figure 1b shows the dependence of the force exerted on a cell (theory) as a function of the indentation for a sinusoidal modulation of the tip-cell distance.

Let us consider an axisymmetric probe that applies a force on a soft sample of thickness *h* deposited on the *xy* plane. The sample rests on a rigid support. The axial deformation *uz* along the perpendicular direction to *xy* plane on a point (,) can be expressed in terms of the pressure distribution *P*z and the Green’s function *G* as (5,49)

(1)

The *G(ρ)* function represents the displacement profile generated by a point force applied on a surface point (*x,y*) at a distance ; *dA* is an element of the area of the contact region. Dimitriadis et al. (5) showed that the *G* function of a finite sample could be expressed as the Taylor series of the ratio ε between the indentation at the center of the contact area *δ* (it coincides with the maximum indentation) and the sample thickness *h* (*ε*=*δ*/*h*)

(2)

where *G0* is the Green function of a semi-infinite sample with the same intensive mechanical properties than the finite sample. For a probe of shape the deformation on a point inside the area of contact is given by

(3)

with . Then the pressure profile can be expressed in terms of a Taylor series as

(4)

The first order term *P*0 represents the pressure profile exerted by the same probe on a semi-infinite sample. The *Pn* terms are the solutions of integral equations (see Supporting Material). Once these integrals are solved, the force exerted on the cell could be recovered by integrating the pressure profile within the contact region.

(5)

A flat-ended cylindrical probe (flat punch) provides a geometry that facilitates the solution of the integral equations to determine the force profiles. It represents our reference geometry for the calculations of the next sections. For that reason, we provide the analytical expression of the *Pn* components for the flat punch (see Supporting Material)

(6a)

(6b)

(6c)

(6d)

(6f)

where *a* is the radius of the punch, *E* is the Young modulus of the sample, ν is the Poisson coefficient; and are numerical parameters that depend on the type of attachment of the sample to the rigid substrate and the Poisson coefficient (5,31). For (this is, outside the contact region), *Pn* are zero.

To determine the force by applying Eq. 5 for other geometries might be very demanding. In general, the integral equations leading to *Pn* are hard to solve. For some geometries, the integrals might not have analytical solutions. In addition, the direct approach cannot be generalized. A change of the probe’s geometry involves solving a new set of integral equations. For those reasons, we implement an alternative method which is equivalent to the direct approach but it is mathematically more manageable. This method is applicable for any axisymmetric tip.

**Bottom effect elastic theory to determine the force exerted by an axisymmetric probe**

The method combines the reciprocal theorem for linear elastic solids and the determination of the pressure profile exerted by a flat punch. The reciprocal theorem developed independently by Betti, Maxwell and Raleigh (50), establishes an equivalence between the work performed by two different pressure fields on the same sample, such as the ones generated by two different AFM probes.

Let us consider a linear elastic body of arbitrary shape. The application of a field of pressures *Pi* (*xj*) on the surface of the body will generate a field of deformations , where *xj* represents the spatial coordinate in the *xyz* space where the deformation is calculated. If we apply a different field of pressures *P\*i* (*xj*), for example, by using a probe with a different geometry, we will obtain a different field of deformations *u\*i* (*xj*). The reciprocal theorem establishes the following equivalence

(7)

where the integral is performed over the whole surface of the body. As a reference probe we use the flat punch because we have already deduced the analytical expressions of the pressure distribution and the deformation. In Eq. 7 the only unknown is the field of pressures *Pz*. The deformations *uz* across the contact region are determined from the probe shape.

To simplify the mathematical derivation, we assume that all the forces exerted on the sample surface are applied parallel to the vertical axis *z*, then the lateral components of the pressure are zero,

(8)

Let us apply the reciprocal theorem for an axisymmetric probe of geometry *f*(*r*) and the flat punch (*fpunch*(*r*)=0) (see Supporting Material)

(9)

which leads to an expression of the force exerted on the sample in terms of the maximum indentation and the contact area as *F*(*δ*,*a*)

(10)

We have assumed that the maximum indentation *δ* and the contact radius *a* are identical for the reference and problem probes. We note that with independence of the probe geometry there is a flat punch that will satisfy the above conditions.

**Force-indentation equations for a flat punch, paraboloid and conical probes**

To obtain the force as a function of the indentation on a single cell, we need to define the geometry of the indenter, to include the cell’s incompressibility (Poisson coefficient νcell = 0.5), to set some boundary conditions such type of cell attachment to the solid support, and to determine the dependence of the contact area as a function of the indentation (finite thickness). The latter is obtained by solving the following (51)

(11)

The mathematics to solve the integral of Eq. 10 for the different geometries involves several intermediate steps (Supporting Material). The intermediate expressions are valid to describe the forces and deformation of any sample of finite thickness on top of a solid support. The particularization for a cell comes from the introduction of the boundary conditions and the cell’s Poisson coefficient.

**RESULTS**

**Flat Punch.** The contact area for a flat-ended cylindrical punch does not depend on the indentation (). By inserting Eq. 6 into Eq. 10, the attachment of the cell to the solid support (boundary condition) and *νcell*= 0.5 we obtain an expression of the force on the cell as a function of the indentation, cell’s thickness and Young modulus

(12a)

with

(12b)

where *F*0 represents the force exerted on a semi-infinite sample. Equation 12a reveals that the force scales with the contact area and the sample thickness. It underlines a counterintuitive result, for a flat punch the *F/F0* ratio does not depend on the indentation value. The key factor is the dependence of the *a*/*h* ratio with *δ*.

**Conical probe.** The shape of a conical probe of semi-angle *θ* is given by

(13)

The application of the reciprocal theorem and boundary conditions leads to

(14a)

with

(14b)

**Paraboloid.** In the case of a paraboloid the shape of the probe is

(15)

Then, the force can be expressed as

(16a)

with

(16b)

The above expression can only be applied for *δ* ≤ *R*, for that reason we call it *Fsphere*. This expression resembles the one deduced by Dimitriades *et al*. (5) but the coefficients that multiply the terms 1/*hn* are different. We have used a non-Hertzian model to express the change of the contact area with the indentation while in ref. 5 the contact area was calculated by using Hertz contact mechanics. Due to that approximation, Dimitriades *et al.* (5) expression underestimates the force applied on the cell (see FS3 in Supporting Material).

**Force-indentation equations for other axisymmetric probes: needles and nanowires**

The same procedure has been applied to deduce the force as a function of the cell properties, indentation and probe geometry for other axisymmetric tips. In particular, we have deduced the force-indentation equations for two of the more interesting probes for mapping nanomechanical properties at high spatial resolution, needles and nanowires. For example, a nanowire is approximated by a flat punch capped with a hemisphere. The force for a nanowire is derived from the flat punch and paraboloid equations by introducing a critical indentation *δc*. This indentation defines the value where the contact radius coincides with the radius *R* of the probe, then

(17)

A needle is approximated by a flat punch capped with a cone,

(18)

**DISCUSSION**

**Consistency of the bottom effect elastic theory**

Figure 2 illustrates the dependence of the force exerted on a cell as a function of time for a tip-cell distance modulation given by

) (19)

The force in equations 12, 14 and 16 are expressed as a sum of terms (1/*hn*). The term corresponding to *n* = 0, represents a sample with infinite thickness. It coincides with the expressions provided by Sneddon (44) for paraboloid, cone and flat punch geometries. We note that Hertz contact mechanics is a particular case of Sneddon’s theory. We have verified that the results described on the following sections are valid for other type of tip-cell distance modulations, in particular, for triangular waveforms.

Figure 2 shows that for those geometries, the force converges as the order of the polynomial is increased. This result represents an internal validation of the theory. We note that a previous attempt to deduce the force exerted by a conical tip on a cell did not converge as more terms were included in the calculation of the force (8) (see FS4 in the Supporting Material).

**Force curves as a function of the cell thickness**

We have calculated the dependence of the force exerted on a cell as a function of its thickness for an indentation of 1 μm for flat punch, conical and parabolic probes (Fig. 3). The cell is simulated withparameters *Ecell* = 4 kPa, *ν*= 0.5. We have considered three values of the cell thickness 2.5, 5 and 10 μm. Those values represent the thickness across different regions of a cell, 2.5 μm near the edges, 5 *μ*m near the nucleus and 10 *μ*m on top of the nucleus. We have also plotted the results for a semi-infinite sample. To provide a complete characterization of these effects the force is plotted as a function of the time and indentation.

The force increases as the sample thickness is decreased. This observation is an unavoidable consequence of three factors. The cell’s Poisson coefficient, cell’s thickness (1-15 μm) and its very small Young’s modulus (0.5-10 kPa). The results illustrate the influence of the stiffness of the substrate on force spectroscopy measurements. The bottom effect elastic corrections decrease as the thickness sample (cell) is increased.

Figure 4 shows the competition between the bottom effect, the indentation and the contact area in the measured force. Force distance and time curves are calculated as a function of the probe’s radii for a nanowire. To visualize the bottom effect contribution we have calculated the force exerted by the same probe on a semi-infinite sample (discontinuous curves). The bottom effect plays a major factor in the determination of the force whenever the contact radius and sample thickness are within the same order of magnitude. In cell nanomechanics, the use of sharp and high-aspect ratio probes significantly reduce the influence of the solid support in the determination of the force.

**Comparison between bottom effect corrections and finite element simulations**

To test the validity of the above expressions we have compared them with finite element method simulations (FEM). In this comparison, the results provided by the FEM simulations are considered to provide the *true* behaviour of a linear elastic and homogeneous material. The numerical simulations were performed by using the COMSOL software (COMSOL Multiphysics, COMSOL AB, Stockholm, Sweden).

Figure 5 shows three of the interfaces simulated by FEM. The interface is formed by the probe, a finite elastic layer attached to a rigid support. The interfaces for a flat punch (Fig. 5a), cone (Fig. 5b) and a paraboloid of radius *R* (Fig. 5c) are shown. In the FEM simulations the probe is considered an isotropic and homogeneous elastic material characterized by a Young’s modulus of 20 GPa. The layer is simulated by a cylinder of length and radius, respectively of, 5 *μ*m and 50 *µ*m. For completeness, we have also simulated the response of a semi-infinite material. In this case, the semi-infinite material is approximated by a thick layer with length and radius, respectively, of 50 *µ*m and 50 *µ*m. The boundary condition for the rigid support implies zero displacements in the in an out-plane directions. The tip-layer distance was modulated by a sinusoidal waveform with a frequency of *fm* = 1 Hz.

The simulations generate what we consider the true force-indentation curves. Those curves are compared with the forces given, respectively, by Eqs. 12, 14 and 16. From the fittings we deduce the Young’s moduli of the finite layer. Those values are compared to the ones introduced in the FEM simulations.

Figure 6 shows the dependence of the Young’s moduli as a function of the indentation/thickness ratio for a sample of thickness *h* = 5 μm. The bottom effect elastic theory enables to recover the Young’s modulus for the three interfaces. The determination of the Young’s modulus provided by the bottom effect theory does not depend on the indentation ratio (Fig. 6). This result is in sharp contrast with the values provided by Sneddon contact mechanics. Sneddon contact mechanics (Hertz’s included) significantly overestimate the value of the Young moduli. The overestimation increases with the indentation for conical (Fig. 6b) and parabolic tips (Fig. 6c) while it remains flat for a punch (Fig. 6a). This happens because the contact area for parabolic and conical tips shows a rapid increase with the indentation. The flat punch represents the opposite case, the contact area does not change with the indentation, as a consequence the error in the determination of the Young’s modulus by using the semi-infinite model does not depend on the indentation (Fig. 6a). The above results illustrate a remarkable property of the bottom effect artefact. On a given cell, the influence of the substrate stiffness increases with the contact area and thus with the size of the probe.

The FEM simulations have been performed by assuming that the surface of sample which is in physical contact with the probe does not have lateral displacements. Those displacements happen in the regions of the sample underneath the contact area. In the supporting material we present the results obtained for a boundary condition that allows lateral displacements in surface of the cell in contact with the probe.

The use of large colloidal tips (41,52) with a radius of several micrometers has been recommended to determine the Young’s modulus of living cells (7,41). Those probes provide an uniform and well defined shape during the interaction with the cell. The above results show that the determination of the Young’s moduli with those probes makes unavoidable the use of a bottom effect elastic theory; otherwise, the accuracy of the Young’s moduli provided by force spectroscopy measurements is highly questionable.

**CONCLUSIONS**

The force exerted by a probe on a living cell is influenced by the stiffness of the solid support. We have developed a bottom effect elastic theory to describe the forces and deformations exerted by a probe on an adherent cell deposited on a rigid support. The theory provides analytical expressions to determine the force as a function of the thickness of the sample, the indentation and the contact area. The theory shows that the force exerted on a cell is augmented by the presence of the solid support. This result is an unavoidable consequence of the boundary conditions existing in an AFM experiment. Despite this effect, the bottom effect elastic theory enables to recover the intrinsic mechanical properties of the cell such as the Young’s modulus with independence of the stiffness of the solid support. The bottom effect elastic corrections decrease as the contact area between the probe and the cell is reduced. This leads to a counterintuitive result, for a given cell, the use of sharper tips reduces the bottom effect artefact.

The theory is general. It can be extended to any axisymmetric tip’s geometry. Specifically, we have deduced analytical expressions for flat punch, conical, paraboloid, needle and nanowire probes. The theory has been validated by using finite element simulations.

**SUPPORTING MATERIAL**

Additional figures and information about the theoretical method and simulations are available at http://www.biophysj.org/

**AUTHOR CONTRIBUTIONS**

P.D.G. developed the theory and performed the numerical simulations. R.G. conceived the project, supervised the theory and the simulations. R.G. wrote the manuscript. P.D.G. and R.G. analysed the data, discussed the results and revised the Ms..

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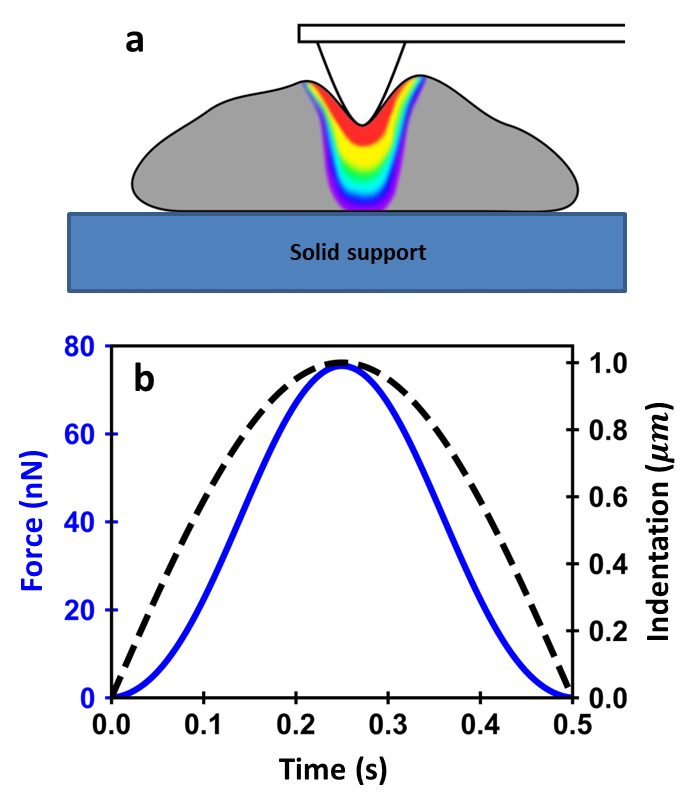
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**Figures**



**Figure 1**. (a). Scheme of the probe-cell-solid support interface in a force spectroscopy measurement. The changes in the color provide a qualitative indication of the stress variation across the interface (stress from high to low, red>yellow>green>blue). (b). Probe’s displacement (black) and probe-cell force (blue) as a function of time for a sinusoidal modulation of *A*=1 μm and *fm*= 1 Hz. Other data: *Ecell*= 4 kPa, *h*=2.5 μm; *R*= 5 μm.

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**Figure 2**. Bottom effect elastic corrections as a function of the approximation order. (a). Force as a function of time for a flat punch with a radius *a*=1 μm. The force curves for the 2nd, 3rd and 4th order approximations are very similar. (b). Force as a function of time for a conical tip with a semi-angle *θ*=45º. (c). Force as a function of time for a parabolic tip of *R*= 5 μm. The numbers indicate that the force is calculated by adding the terms until that order, for example, for a conical indenter, *Fcone*(0th)=*F*0=*F*Sneddon-cone, *Fcone*(1st)=*F*0+*F*1, *Fcone*(2nd)=*F*0+*F*1+*F*2 and so on. Other data: *Ecell*= 4 kPa, *h*= 2.5 μm; *A*=1 μm and *fm*= 1 Hz.

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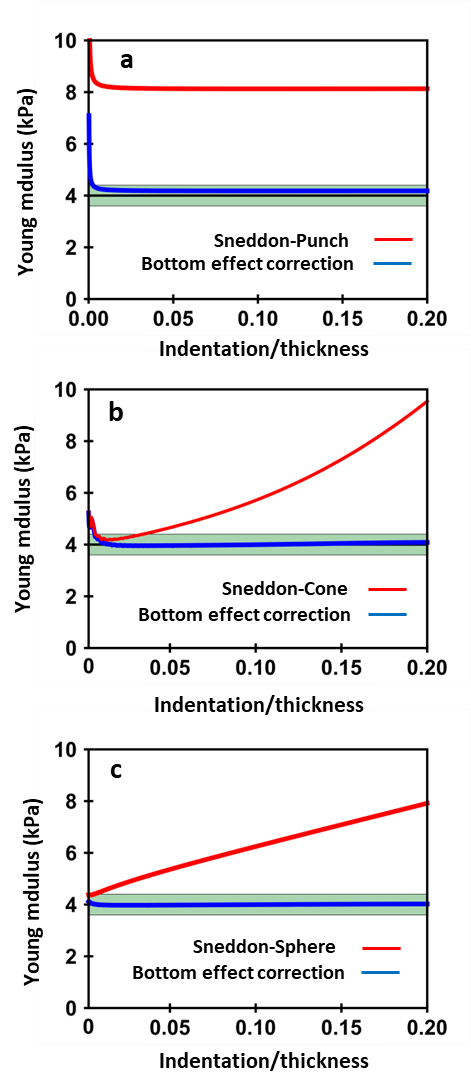
**Figure 3.**  Force curves as a function of the sample thickness. The force is plotted as a function of time and indentation for flat punch, parabolic and conical probes. (a) Side view of the flat punch-cell-solid support interface. (b). Force-time curve for a flat punch (*a*= 1 μm). (c). Force-indentation curve for a flat punch (*a*= 1 μm). (d). Side view of the cone-cell-solid support interface. (e). Force-time curve for a cone (semi-angle *θ*=45º). (f). Force as a function of indentation exerted by a cone (*θ*=45º). (g) Side view of the paraboloid-cell-solid support interface. (h). Force-time curve for a parabolic tip (*R*= 5 μm). (i). Force-indentation curve for a parabolic tip (*R*= 5 μm). The force curves have been calculated with the 4th order approximation. Sinusoidal modulation of *A*=1 μm and *fm* = 1 Hz; *Ecell*= 4 kPa. Thickness of the sample, from top to bottom: 2.5 μm, 5 μm, 10 μm and semi-infinite.

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**Figure 4.** Force curves for nanowire probes. (a). Side view of nanowire-cell-solid support interface. (b). Force-time curve for nanowires of *R*=1 μm, 500 nm and 50 nm. (c). Force-indentation curve for different nanowires. The discontinuous lines show the force curves obtained for the same probe applying a force on a semi-infinite sample. For the sharpest tip, the force curves given by the semi-infinite model and the bottom effect elastic theory are nearly identical. Layer parameters, *Ecell* = 4 kPa, *h*=2.5 μm, semi-infinite *Ecell* = 4 kPa.

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**Figure 5.** Finite element interfaces. (a). FEM scheme of the interface formed by a flat punch on top of finite elastic layer deposited on a rigid solid support. (b). FEM scheme of a flat punch on top of a semi-infinite linear elastic sample. (c). FEM scheme of the interface formed by a conical tip on top of finite elastic sample deposited on a rigid solid support. (d). FEM scheme of a conical tip on top of a semi-infinite linear elastic sample. (e). FEM scheme of the interface formed by a parabolic tip on top of finite elastic sample deposited on a rigid solid support. (f). FEM scheme of a parabolic tip on top of a semi-infinite elastic sample. The sample either finite or semi-infinite has an *Ecell* = 4 kPa. The simulated systems have cylindrical symmetry around the vertical axis (red line).



**Figure 6**. Determination of the Young modulus from the bottom effect theory and Sneddon contact mechanics. (a). Flat punch of *a*=2.5 μm. (b). Conical tip of *θ*=76º. (c). Parabolic tip of *R*= 5 μm. The elastic moduli determined by the bottom effect (blue line) and infinite thickness theories (red line) are plotted as a function of the indentation/thickness ratio (*h*=5 μm). The bottom effect elastic corrections have been calculated by using the 4th order approximation. The force-distance curves have been generated by FEM simulations for a 5 μm thick sample with Young’s modulus 4 kPa. The curves are fitted with the expressions for the flat punch, cone and parabolic tips. The green stripe shows the values that lie within a 10% window from the Young’s modulus of the sample (4 kPa).