Ref. 1

This manuscript describes highly innovative and ambitious work aimed at providing insight into prebiotic behaviour. The manuscript describes a robotic system capable of implementing a genetic algorithm on a simple chemical system. The chemical system consists of droplets of five components in water, four of which are automatically and iteratively varied, with the robot selecting for specified behaviour. The outcome of this is a series of mixtures which produce interesting physical behaviour, such as droplet stability, motility, or division. This technology could, in principle, have a significant impact on research into protocells, compartmentalisation, and 'systems chemistry' by allowing for the automatic and efficient exploration of chemical space for hard-to-predict behaviours such as those described above.

Overall, I would like to see this work published, but I have serious concerns about the way it is currently written and I have several questions which will probably be shared with the intended audience. The paper is clearly aimed at chemists, specifically systems chemistry types and the origins of life communities. I suspect both groups (and a more general audience) could all enjoy reading this work and be stimulated by this research - here a (potentially powerful) approach has been outlined and this will no doubt tweak the imaginations of chemists in a variety of fields. It is also worth highlighting that the type of behaviour that the authors seek to observe, and the overall scientific questions that they aim to address, are difficult problems of high general interest.

Somewhat superficially, the paper is written from a 'rigorously' prebiotic perspective, and I'm not sure it works. Terms like assisted evolution are obviously appropriate but I found myself cringing as phenotypes, genomes and mutations were discussed. I don't think that the terms were used incorrectly, just that it all seemed a little much.

Similarly, the article switches from discussions that are "prebiotic" to "chemistry" to "robotics" to "fitness landscapes" and it just doesn't feel like a balanced or intelligible story, I was actually surprised when I got to "in summary" as I was anticipating some kind of analytical and mechanistic discussion.

Too much of the paper deals with methodology / the evaluation of protocols / statistical analysis and not just a discussion of what chemical phenomena are being observed / explanations. I feel that the much of the methods (ie most, especially the discussion surrounding figure 3 and the figure itself) should be moved to the SI as it is discussed in the methods summary anyway. What chemists will be interested in is not the methodology of selection --- it is a robot doing the selection after all, so the 'selection' process itself cannot possibly be prebiotically relevant or chemically interesting --- but 1) the fact that it can be done and 2) how the composition of chemicals changes the behaviour, and 3) mechanistic insight into how composition controls behaviour.

If the authors had studied their 'evolved' droplets and learned what their robot had actually selected for, and thereby gained some insight into the chemistry, it would significantly improve the papers appeal to a chemical audience - I suspect that the authors have this information (see Si section S.5.3, Fig 24) and would strongly encourage them to expand on this and to incorporate an analysis front and centre in the main text.

It is also not clear exactly what is being achieved by the 'selection' process in some cases - for example, in SI video 6 'movement' ¬- is the behaviour of the 1st generation system actually that different than in generation 20? Clearly things have changed in the middle, but to untrained eyes the outcome looks remarkably similar to the starting point. Some explanation / discussion is clearly needed.

Other aspects

• Does the paper stand out in some way from others in its field?

The technology described herein is remarkable in itself, and represents a potentially powerful and intelligent approach to systems chemistry. It does represent a different approach to protocell research than has been undertaken previously.

• Are the claims convincing? If not, what further evidence is needed?

The key claims - that complex behaviours can be observed by subjecting a simple mixture to selection, and that this robot is a powerful means to this end - are convincing and the experiments herein are sufficient to demonstrate this. Some secondary claims that are not central to the paper are problematic.

• Are there other experiments that would strengthen the paper further? How much would they improve it, and

how difficult are they likely to be?

No, but reanalysis of some experiments is necessary. The authors should discuss SI section S.5.3 in the text, and then in some detail discuss the composition of (at least) two 'end product' droplets, and at least try to understand (or present hypotheses of) how their differing compositions drive their different behaviours. The authors briefly address this on page 11 of the manuscript in the context of fitness landscapes (but honestly I find the analysis almost unintelligible) and do not attempt to chemically rationalize the behaviour.

If the authors can understand why, for example, octanoate inhibits movement and division but is necessary for vibration (or any similar insight), this will be more interesting to chemists and will demonstrate the power of this technique to go from simple mixtures to complex behaviour to chemical insight.

• Are the claims appropriately discussed in the context of previous literature?

Generally, yes. The exception is the use of the term 'evolution first hypothesis'. This term is not defined by the authors, but I infer that they mean a scenario in which pre-living systems undergo some form of natural selection or evolution before the onset of life. This is not a new idea, and the authors should elaborate on the use of this term and discuss related ideas, or if they mean something different, specify.

• Is the manuscript clearly written? If not, how could it be made more clear or accessible to non-specialists?

No, there is a general trend towards imprecise or convoluted wording. Some topics which are not really 'chemistry' are not described in an accessible manner. Particularly, the authors should provide a brief comment on what fitness landscapes are and how to interpret them. They should give a concise plain-English explanation of their genetic algorithm set-up (pages 8-10) and move the rest to the SI.

The authors should more clearly contrast their initial exploration of a limited space of 225 combinations (page 9) with their subsequent selection experiments (with a chemical space of ~107 combinations). A short sentence at the end of page 8 or thereabouts would make the contrast explicitly clear from the start.

The authors should clarify why they do not vary TTAB in addition to the other four components.

• Could the manuscript be shortened?

Yes. The introduction is longwinded.

• Have the authors done themselves justice without overselling their claims?

Yes. But two quibbles: Are these drops really protocells? A protocell has some metabolic or genetic content contained within. This in no way diminishes their potential importance and utility as models for protocell dynamics, of course.

Second "This highlights the plausibility of evolutionary forces acting at a very early stage of abiogenesis" (page 12). While the use of artificial selection and exploration of fitness landscapes here is interesting, it is not at all clear that these systems could be subject to natural selection or persist (as multiple generations) over evolutionary time. This claim should be removed.

• Have they been fair in their treatment of previous literature?

With the exception of the 'evolution first' hypotheses, yes.

• Have they provided sufficient methodological detail that the experiments could be reproduced?

The SI is superb.

• Is the statistical analysis of the data sound?

This is beyond my expertise, but it appears appropriate.

Ref. 2

Gutierrez et al present the use of a robotic platform to formulate oil droplets that show a variety of behaviours (dissolution, splitting, movement) upon placement in water. The authors discuss an iterative procedure for identifying composition with certain properties.

It is known that oil droplets in water can show interesting behaviours due to combined effects of surface tension, gradual dissolution of components, acid-base equilibria. The droplets used here show even richer behaviour because of the use of mixtures, which has unpredictable effects on physical properties of individual components.

The paper, however, is not about gaining a detailed insight into the behaviour of oil droplets. Instead, the authors are interested in creating a system that undergoes some type of evolution. I am afraid I don't think the authors make a convincing case.

Let us consider the starting premise (p2). 'We set out to take the simplest model of a protocell, which is characterized by an interface or membrane separating the inside from the outside...'

using this definition, the authors have not made protocells (even though the title and the whole document insist otherwise).

Second part of the same sentence: 'how to design a robotic system to facilitate the evolutionary process...'

I have thought about this sentence and I don't think it makes a scientifically justifiable starting point. I very much agree that making a system that shows evolution is a grand challenge for physical sciences. But by using a machine to do the evolution for you, the whole challenge is gone. As stated later on the same page: ... a robotic assistant could be used as a 'fitness crutch' (no idea what this means!!!) and ' individuals that are not capable of sustaining chemotrophic replication (again: what does this really mean??) and 'In this way the robot embodies the evolutionary process...' completely misses the point about evolution. There is no appreciation of what evolution in a biological sense really means. One has a genome that stores information and through sexual selection, the fittest genomes are selected, while the combination with mutations allow for evolution. really, this paper is about a robot mixing formulations for oil droplets. There is no link to evolution whatsoever (and redefining or inventing terms is not going to help).

On page 3/4 it is 'hypothesized that emergent behaviour will result from this coupling'. What does this mean. What behaviour. How do things emerge? The same vague language is used again on page 6 '.. allowing us to explore the potential for the discovery of emergent properties, complex behaviours and ...embody evolution'... Again, what complex behaviours?? What does 'embody evolution' mean?? A compositional genome????

Then on page 10, a detailed description is given about a method to find mixtures of oils with interesting properties. Essentially, this is the same routine industry uses to optimize formulations in food, shampoo, cosmetics, fuel, etc. The application to use such a routine to discover regions of interest for droplet properties is very nice. Not sure if one would call it a fitness landscape as in Fig 5. Also not sure what 'approximate but accurate' means.

Page 11: .. this is similar to the concept of... pleiotropy'. No it is not. In pleiotropy, there is a mutation. There are no mutations here, and in Figure 5, the change in concentration of a component is investigated.

On page 12: the authors draw the conclusion that their work ' highlights the plausibility of evolutionary forces acting at a very early stage of abiogenesis.. ' No, really. Where exactly did the authors begin to get even close to abiogenesis???

In conclusion, I don't think this manuscript presents a coherent body of work with conclusions supported by experimental evidence nor is it based on a sound scientific hypothesis.

This is not to say that the observed properties of the oil droplets are not interesting. They are fascinating and intriguing and much can be learned from studying structure-property relationships (which the authors have not yet done, but they could do this in combination with the computational tools presented). Such a study would be a very different paper altogether and I look forward to reading it.

Ref. 3

As the authors say, the questions around evolution are profound, but I really doubt that the present manuscript can be helpful in that respect. The authors constructed a completely artificial system, programmed to do what they asked for, including a manipulating robot, and with droplets which are indeed rather far away from biological reality. It is a self-consistent system, somebody may say an intellectual game, well-conceived and the work is carried out with good professionalism. For this reason, I feel that it should be published, but not in a chemical or a biology journal. It belongs to artificial intelligence, and should find place in a journal of this family.

Ref. 4

Review: Assisted Evolution of Protocell Droplets in a Chemo-Robotic Platform
The authors combine 3D printing, robotic systems and evolutionary algorithm to create and (with assistance) evolve chemical droplets with specific characteristics, by exploring the chemical composition space of such droplets. Their methodology allows them to systematically scan the compositional space and aim for emergent characteristics of the droplets. While in recent years the composition of the molecules comprising a vesicle has been shown to effect their physical properties (such as permeability [Astrobiology 9, 979 (2009)] and structure [Bioch. Bioph. Acta Biomembranes 1798 (2010)]), the present work advances this by the automated assisted discovery and by exploring more components (four). Importantly, the present work finds such characteristics optimally arise for non-trivial compositions (a fitness peak in the middle of the Fig. 5 panels, not necessarily close to the boundaries). The authors further suggest these droplets as a model of protocells in the origins of life (OOL). On the whole the work is original and provides a novel approach to understanding early evolution.

Major comments:
1) In the abstract it is written “Theories involving RNA, proteins, metabolism, or protocells”. A protocell is an entity with very basic life faculties, indeed the “first evolvable system” (quoting from the text). A protocell, in the eyes of many, is a general term that may involve one or more of the components mentioned (RNA, proteins, metabolites), and thus is not on par with them. What is missing is “compartments”, the droplets described here being an example. The novelty that needs to be highlighted in the abstract and elsewhere in the paper is that within the realm of early compartments, also related also to “Lipid first” scenarios, the author’s pursuit belongs to a research direction ascribing information content and evolvability to entities composed solely of amphiphiles, without DNA, RNA or proteins. Along similar lines, it is inappropriate to say “we set out to take the simplest model of a biological cell, a protocell. This work is more about showing that oil droplets are models for protocells!
2) This paper deviates considerably from the widely accepted notion that a gene is a string of chemical “letters”, and a genome is a collection of genes. Here a gene is a chemical substance; in fact a number that indicates its proportion in a mixture, and a genome is a collection of such numbers. The authors should provide literature on the history of this dissenting view and lists
formal aspects. In the same vein, references should be provided to earlier work that addresses compositional genomes (PNAS 97:4112, 2000; Bioessays 28:399, 2006).
3) Some central aspects of the methods used are not provided in the main text so as to be self-contained and allow the reader to follow accurately what has been done. An example of obscure description is the text “The droplet formulations are based upon the following reagents ((1-octanol, 1-pentanol, diethyl phthalate (DEP), dodecane and dilutions of octanoic acid in one of the other oils (typically 20% v/v)). Another example is the text “using a GA”, which presumably stands for Genetic Algorithm, elsewhere in the paper mentioned as “evolutionary algorithm”, quite confusing. A third instance is the sentence “To explore the possibility of evolving protocells
for different traits, behaviors, or fitness, a subsequent series of experiments employing an evolutionary programming approach within our chemo-robotic platform.” – missing “was performed”?. A last example is “…”This is the concept of biological concept…”. There are quite a few other examples (some more indicated below), so the Manuscript requires extensive editing for clarity and language.
4) Not sufficient analyses are provided to address the robustness of the results: what is the evidence that repeating the entire set of experiments would lead to similar conclusions?
5) At the bottom of Page 8 it appears as if the evolutionary algorithm acts upon an 8 long binary vector that signifies the relative amounts of the 4 compounds in the droplet. Later, it seems to be indicated that what gets mutatated/recombined is a vector of 4 numbers, this needs to be clarified. In general, the description at the bottom of page 8 is far from the exactitude required to describe the matters involved. The text about the actual numerical values involved is unclear, as in one place in the manuscript normalization is not indicated and in another (why separate fragments?) the term “constrained to 1” is used, presumably indicating normalization. Also, what are the initial genome values?

6) The authors describe 9 phenotypic behaviors and 3 phenotypes used for fitness computations. It is necessary to clarify the relations between these two groups and the rational for selecting the three out of nine.
7) The text: “Over 21 generations the fitness values showed significant statistically values (Fdiv=104.1, Fmot =75.9, Fvib =43.6 and p< 10-13 for all the fitness values…” is unclear in terms of language and methodology. Are the F’s actual values or changes thereof?
8) The modelling procedure (middle of page 11) should be explained in some more detail in the main text.
9) A better discussion is required for the choice of 5 compounds in a grouping arrangement of 1,2,3,4 and 1,2,3,5, and of exactly 4 droplets.
10) Fig. 5 misses quite a few points of detail: a color bar is needed (and what is black?); “a fitness landscape with four cells (3-faces)” and “one chemical to the remainder” are unclear; How were the specific projections decided?
Minor comments:
11) “…the presence of epistasis (whereby the interactions between mutations can have separate effects from the individual effects of the constituent mutations)”: “Separate” is inappropriate.
12) Acronyms need to be spelled out (GA, fps etc).
13) “…a one minute video was captured twice for each protocell formulation” vs “Each full evolutionary experiment was carried out over twenty one generations and repeated three times in total” needs clarification, and better written all in the same paragraph.
14) There is an instance of “Figure XX” in the supporting information. This may mean a scan of the consistency of all figure names.

Ref. 5

In this manuscript, the authors describe the design of a robot and accompanying driver software that instructs the device to generate mixtures of four organic liquids and deposit droplets of the mixtures on the surface of an aqueous solution of surfactant. The "behavior" of the droplets is then video recorded and analyzed against recognized or desired responses. After this analysis, the robot generates a slightly different mixture, re-runs the experiment, and judges the effect on the behavior. In this manner, the system explores a variety of mixture ratios to maximize the nature of the response. The entire system is used to simulate evolution, with the robot serving to overcome the inability of the droplets to undergo error-prone replication. Behaviors that the authors observe for the droplets include translation, vibration, and "explosion" (among others).

After a careful reading of the manuscript and review of the supplementary information, I feel the paper should be rejected by Nature Chemistry on the grounds that the work is not especially suitable and scientifically interesting to an audience of chemists.

I have no serious concerns over the construction of the system or the results described by the authors. It is a neat piece of work and the robot is impressive (to a chemist). The videos that accompany the paper in the supplementary information are well produced, captivating, and very effective tools for communicating the classes of behaviors observed by the authors. Most of my concerns regarding this manuscript pertain to relevance:

-- Is this paper especially relevant to evolution and important in this regard?
-- Is this paper relevant to chemistry and the origin of life?
-- Is this paper relevant (or best suited) for a readership of chemists?

In terms of the relevance to evolution, the system constructed by the authors is most certainly relevant. The authors have constructed a chemical system, controlled by robot and evaluated by image analysis, that applies an exogenous fitness function to model evolution. This concept is neat--very neat--but there is no compelling argument that there is anything special about this evolutionary process. The authors raise the general idea of the Marangoni Effect, but they seem to actively avoid a more thorough discussion the surface effects at play and how changes in concentration of these four particular components would change the behavior of the system. If the reader is to believe that the behavior of the droplets is especially unexpected, shouldn't the observations be accompanied by an analysis of what behavior we should have expected to see? The main contribution of the paper is in engineering a new platform to study evolution, not in contributing to how we understand evolution itself.

The main argument against the relevance of this system to the origin of life is that the system suffers from a resounding lack of prebiotic significance. The four compounds chosen to construct the droplets have no special prebiotic relevance. The idea of natural selection by computer-aided image analysis has no prebiotic relevance. And finally, replication by robot has no prebiotic relevance. The system is highly contrived: a

computer picks out properties it has been programmed to look for, and it creates new generations as carefully controlled mixtures of molecules from pure stock samples. Saying a droplet of oil on a layer of water is a "protocell" doesn't offer any compelling reason to think of it as a protocell. Aside from the idea that the origin of life on Earth probably entailed some sort of chemical evolution, any further connection of this paper to the problem is weak. While the authors allude to relevance of their work to OoL chemistry, the data and discussion do not support this case in any meaningful way.

Finally, Nature Chemistry is a journal primarily read by chemists. I believe that the manner in which the manuscript is written makes it hard for a typical chemist to understand what is going on and why it is interesting. As discussed before, the surface phenomena at play in the system are not discussed with any degree of thoroughness or sophistication. The paper itself focuses on (i) the design of the robotic system and (ii) a discussion of the relevance of the droplets to evolution and emergent behavior. If these are the foci, I think the authors should submit the manuscript to a journal that either specializes in robotics, peculiar evolution, or complexity/emergent behavior. The analysis of the chemistry involved in the system--how you explain what is happening on a molecular basis--is not a focus of the manuscript.

So, basically, I find this paper interesting and neat. I don't have any big problems with the robot itself or the experiments. I would have liked for the authors to include a more sophisticated, clear, and molecule-focused discussion that explained the observed behavior of the droplets. Furthermore, I was not won over by the sales pitch that this work is especially new or relevant to the origin of life or early evolution. I recommend that Nature Chemistry reject this manuscript, and that the authors revise their writing and resubmit the work to a journal related to evolution or robotics, where experts in these fields may evaluate it.